

Direct Actions of Kit-Ligand on Theca Cell Growth and Differentiation During Follicle Development*

JEFF A. PARROTT AND MICHAEL K. SKINNER

Reproductive Endocrinology Center, University of California, San Francisco, California 94143-0556;
and Center for Reproductive Biology, Department of Genetics and Cell Biology, Washington State
University, Pullman, Washington 99164-4234

ABSTRACT

The direct actions of kit-ligand/stem cell factor (KL) in developing ovarian follicles were investigated. Previous studies have shown that granulosa cells express KL that can support oocyte development. The current study demonstrates that KL can also act directly on theca cells to promote cellular growth and differentiation. Through RT-PCR analysis it was shown that bovine granulosa cells express KL, and theca cells express the receptor c-kit. Bovine theca interna cells were isolated and cultured in serum-free conditions to study KL actions. KL stimulated theca cell growth in a dose-dependent manner as measured by [³H]thymidine incorporation into DNA when cells were cultured under subconfluent conditions. KL had no effect on theca cell androstenedione or progesterone production under these growth-permissive conditions. In contrast, KL stimulated theca cell androstenedione production but had no effect on progesterone production when theca cells were cultured under confluent (non-growth-permissive) conditions. Estradiol (10⁻⁷ M) and human CG (100 ng/ml) were used as controls and regulated theca cell steroid production at any cell density. These results demonstrate that KL can directly stimulate theca cell growth and steroid production during follicular development. The observation that KL stimulated androstenedione produc-

tion but not progesterone production suggests that KL promotes a follicular phase differentiated state in theca cells. The potential regulation of KL and c-kit expression during follicular development was studied using a specific quantitative RT-PCR procedure. Total RNA from granulosa cells (for KL) and theca cells (for c-kit) was examined from small (<5 mm), medium (5–10 mm), and large (>10 mm) size follicles. Steady state levels of KL messenger RNA were highest in granulosa cells from large size follicles and lowest in small and medium size follicles. No differences were observed in the steady state levels of c-kit messenger RNA in theca cells from small, medium, or large size follicles. The observation that KL expression is highest in large size follicles suggests that KL may be important for increased growth and steroid production in large and dominant follicles. Observations demonstrate that KL can dramatically alter theca cell function and support the hypothesis that local granulosa-theca cell interactions play an important role in regulating cellular function within ovarian follicles. This study identifies KL as the first granulosa cell-derived growth factor that can directly stimulate theca cell growth and androstenedione production in the absence of gonadotropins. (*Endocrinology* 138: 3819–3827, 1997)

MESENCHYMAL-EPITHELIAL cell interactions between theca cells and granulosa cells are essential for ovarian follicular development. Mesenchymal-derived theca cells produce a number of factors that act locally to regulate the growth and differentiation of adjacent epithelial granulosa cells (1–3). These cell-cell interactions ultimately determine the fate of developing ovarian follicles that undergo atresia or fully develop to ovulation. Granulosa cells surround the developing oocyte, providing a critical microenvironment for follicular growth. In addition, granulosa cell feedback on the surrounding theca cells helps regulate growth and differentiation of ovarian somatic cells (4). It has been shown that granulosa cells produce kit-ligand/stem cell factor (KL) that promotes oocyte development in the ovarian follicle (5–10). *In situ* and immunohistochemistry experiments in the rodent demonstrated that the receptor c-kit is highly expressed in oocytes, supporting the role of KL in granulosa cell-oocyte interactions (5, 7, 11–13). Although it is clear from these experiments that c-kit is also expressed in

theca cells in developing follicles (6, 7, 12), the direct role of KL in regulating theca cell function has not yet been addressed.

The KL [also named stem cell factor (14), mast cell factor (15), or steel factor] and its tyrosine kinase receptor c-kit are encoded at the steel (Sl) and white spotting (W) loci of the mouse, respectively (16–22). Both Sl and W mutations cause defects in melanogenesis, gametogenesis, and hematopoiesis at several stages of embryonic development and adult life (23–28). During male and female embryonic development, KL and c-kit are essential for germ cell migration (29–33). In the adult, KL and c-kit are important for follicular development in the ovary and survival/proliferation of type A spermatogonia in the testis (6, 34). Several KL and c-kit mutations have been described with many gonadal phenotypes. The ovaries of adult mice carrying the steel-panda (Sl^{Pan}), steel-contrasted (Sl^{con}), or steel-t (Sl^t) mutations contain predominantly small follicles whose development is arrested at the stage that theca cells organize around the follicles (35–37). This lack of follicle development and subsequent infertility have been widely attributed to a defect in granulosa cell-oocyte interactions via KL/c-kit. However it is possible that these mutations arrest follicular development by disrupting granulosa cell-theca cell interactions. Terada *et al.* (38) found that ovaries from suckling Sl/Sl^t mice do not produce androgens in response to LH, suggesting a defect in theca cells.

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Address all correspondence and requests for reprints to: Michael K. Skinner, Center for Reproductive Biology, Department of Genetics and Cell Biology, Washington State University, Pullman, Washington 99164-4234. E-mail: skinner@mail.wsu.edu.

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Kuroda *et al.* (37) suggested a possible stromal cell/theca cell defect in the same mutant mice. These studies raised the possibility that granulosa cell-derived KL may promote follicular development by directly regulating theca cell function.

The current study used bovine ovaries to examine the potential role of KL to directly regulate theca cell growth and functional differentiation. Experiments are also presented that evaluated the regulation of KL and c-kit messenger RNA (mRNA) expression during follicular development. These experiments establish KL as the first granulosa cell-derived growth factor that stimulates theca cell function in the absence of gonadotropins. These observations help gain an understanding about the local feedback mechanisms that regulate mesenchymal-epithelial cell interactions (*i.e.* theca-granulosa cell) in the ovary.

Materials and Methods

Tissue isolation and serum-free cell culture

Bovine ovaries were obtained from young nonpregnant cycling heifers less than 10 min after death. Ovaries were delivered fresh on ice or immediately frozen at -70°C and delivered on dry ice by Golden Genes (Fresno, CA). Granulosa cells were isolated by microdissection from fresh tissue and cultured as previously described (39). Theca interna layers were then microdissected away from the follicle wall and enzymatically dispersed with 2 mg/ml collagenase (Sigma, St. Louis, MO) in $\text{Ca}^{++}/\text{Mg}^{++}$ -free buffer (40). Cells were immediately plated in 24-well culture plates and maintained at 37°C in a 5% CO_2 atmosphere in the absence of serum. The indicated cells were treated with estradiol (10^{-7} M) (Sigma), human CG (hCG) (100 ng/ml or 4010 IU/mg) (Calbiochem, La Jolla, CA), recombinant human KL (50 ng/ml) (R & D Systems, Minneapolis, MN), or epidermal growth factor (EGF) (50 ng/ml) (Gibco BRL, Gaithersburg, MD). Theca interna cell preparations obtained by this procedure have been characterized cytochemically to contain less than 5% contamination with endothelial and/or granulosa cells (40).

Growth assays

Cell growth was analyzed by quantitating [^3H]thymidine incorporation into newly synthesized DNA. Theca cells were plated at subconfluent densities (<1 million cells/ cm^2) in 0.5 ml DMEM containing 0.1% calf serum. After 24 h, the cells were treated with no growth factor (control), 10–50 ng/ml KL, or 50 ng/ml EGF as a positive control. Cells were plated for 24 h, then treated for an additional 24 h or 48 h. After treatment, 0.5 ml DMEM containing 2 μCi [^3H]thymidine was added to each well, and the cells were incubated for 4 h at 37°C followed by sonication. The quantity of [^3H]thymidine incorporated into DNA was determined as previously described (41). Data were normalized to total DNA per well using an ethidium bromide procedure, described previously (40). Under these subconfluent culture conditions, approximately 0.5–1.5 μg DNA was detected per well. Values of [^3H]thymidine incorporation were generally greater than 2×10^3 cpm/ μg DNA.

Steroid assays

Steroid production by theca cells was determined by quantitating androstenedione and progesterone accumulation in the culture medium. Fresh theca cells were plated at subconfluent densities (~ 0.5 –1 million cells/ cm^2) or at confluent densities (~ 3 –4 million cells/ cm^2) in 1 ml serum-free Ham's F-12 medium containing 0.1% BSA. Cells were immediately treated with 10^{-7} M estradiol, 100 ng/ml hCG, or 50 ng/ml KL and cultured for 72 h. At the end of the culture period, the medium was collected and assayed for androstenedione and progesterone using the RSL ^{125}I -androstenedione kit and the ImmunoChem ^{125}I -progesterone kit, respectively (ICN, Costa Mesa, CA). The sensitivities of the steroid assays are 0.01 ng/ml for androstenedione and 0.01 ng/ml for progesterone. The cells were cultured for an additional 4 h in 0.5 ml DMEM medium containing 0.1% calf serum and 2 μCi [^3H]thymidine to

determine whether or not the cells were proliferating. Under subconfluent culture conditions, [^3H]thymidine incorporation values were generally greater than 2×10^3 cpm/ μg DNA, indicating that the cells were readily entering the cell cycle. Under confluent culture conditions, [^3H]thymidine incorporation values were generally less than 2×10^2 cpm/ μg DNA, indicating that the cells were contact inhibited and not proliferating. The observation that [^3H]thymidine incorporation in confluent cell cultures was at least 10-fold less than in subconfluent cell cultures validates the use of these culture conditions as nongrowth permissive and growth permissive, respectively. All steroid data were normalized to total DNA per well. Under these culture conditions, approximately 0.5–2.5 μg DNA (subconfluent densities) and 6–10 μg DNA (confluent densities) was detected per well.

Preparation of RNA and PCR

Follicles were dissected from the bovine ovaries and separated into pools of small (<5 mm), medium (5–10 mm), and large (>10 mm) size follicles. Granulosa and theca cell total RNA was extracted from each pool of samples using a guanidium thiocyanate procedure followed by centrifugation through a cesium chloride gradient (42). Alternatively, total RNA was prepared using the RNA-Stat 60 kit (Tel-Test, Friendswood, TX). For qualitative analysis of gene expression, 10 μg total RNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Gibco BRL) at 37°C for 1 h using oligo(dT) $_{12-18}$ primers (Gibco BRL). This complementary DNA (cDNA) template was amplified by PCR using specific bovine primers for KL, c-kit, or the constitutively expressed gene cyclophilin (IB15). The KL primers were 5'-GGA CAA GTT TTC GAA TAT TTC TGA AGG CTT GAG TAA TTA TTG-3' (5' primer, 42-mer) and 5'-GGC TGC AAC ACG GGG TAA CAT AAA TGG TTT TGT GAC ACT GAC-3' (3' primer, 42-mer), which generated a specific 315-bp KL PCR product from bovine granulosa cells. These KL primers are designed to specifically amplify the longer KL transcript, which codes for the secreted form of the factor. Experiments using a different 3' KL primer that amplifies both the soluble (KL1) and membrane-bound (KL2) forms of KL demonstrated that bovine granulosa cells primarily express the soluble form of KL (Fig. 1B). This alternative KL primer is described below in the quantitative RT-PCR method. The c-kit primers were 5'-GTT CAT GTG TTA CGC CAA TAA CAC TTT TGG ATC AGC AAA-3' (5' primer, 42-mer) and 5'-TTC AGT CCC TTT TAA TCT GGT TAG ATG AAG TTC ATT-3' (3' primer, 36-mer), which generated a specific 298-bp c-kit PCR product from bovine theca and stromal cells. The primers for cyclophilin (IB15) were 5'-ACA CGC CAT AAT GGC ACT GGT GGC AAG TCC ATC-3' (5' primer, 33-mer) and 5'-ATT TGC CAT GGA CAA GAT GCC AGG ACC TGT ATG-3' (3' primer, 33-mer), which generated a specific 105-bp product from all cell types demonstrating the integrity of the RNA samples. Amplification was performed with AmpliTaq DNA polymerase (Perkin Elmer, Foster City, CA) for 35 cycles using the following conditions: 0.8 μM each primer, 100 μM deoxynucleotide triphosphates (dNTPs), 1.5 mM Mg^{++} , and 1.25 U Taq polymerase in 50 μl total volume. PCR products were visualized by UV illumination (312 nm) of 2% agarose gels stained with ethidium bromide.

The KL, c-kit, and IB15 PCR products were subcloned into the Bluescript plasmid (Stratagene, La Jolla, CA) at the *Sma*I site. Each subclone was sequenced in both directions and confirmed to be bovine KL, c-kit, and IB15. These subclones were used as standard templates in the quantitative PCR procedure below.

Quantitative RT-PCR assays

Steady state levels of KL, c-kit, and IB15 mRNAs were measured using a specific quantitative RT-PCR assay for each gene. The primers used in this quantitative analysis of KL, c-kit, and IB15 were the same as described above except for KL (see below). Before RT, tubes containing total RNA and specific 3'-primers were heated to 65°C for 10 min to facilitate denaturing and cooled to room temperature to facilitate annealing. Total RNA (1 μg) was reverse transcribed for 1 h at 37°C using the following conditions: 1 μg total RNA, 1 μM specific 3'-primers of interest (up to four different primers including IB15), 0.1 mM dNTPs, 10 mM DTT, 40 U RNase inhibitor (Promega, Madison, WI), and 200 U Moloney murine leukemia virus reverse transcriptase (Gibco BRL) in 40 μl RT buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl_2). After

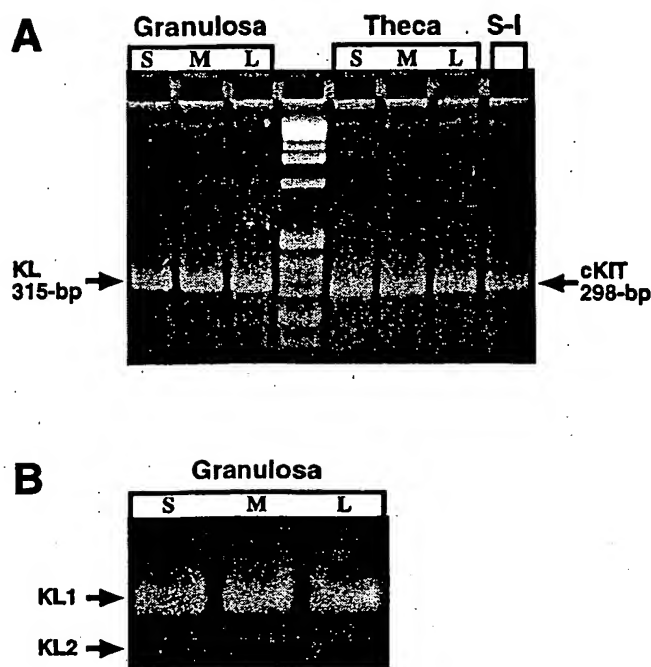


FIG. 1 Expression of KL and c-kit mRNA in bovine ovarian follicles. RT-PCR analysis was performed with 10 μ g total RNA. Amplification was nonquantitative. A, Specific bovine primers for KL or c-kit were designed to generate 315- and 298-bp PCR products from bovine granulosa, theca, or stromal-interstitial cell cDNA template. B, Specific primers were designed to amplify soluble (KL1, 452-bp) and membrane-bound (KL2, 368-bp) forms of KL. KL1 PCR products were predominantly detected in bovine granulosa cells. Standard DNA ladder (1 kilobase) was used for size determination (unlabeled lane). S, small follicles; M, medium follicles; L, large follicles; S-I, stromal-interstitial cells. Data are representative of at least three experiments.

1 h, samples were heated to 95 C for 5 min to inactivate the reverse transcriptase enzyme. Samples were immediately diluted 2.5-fold, and carrier DNA (Bluescript plasmid, Stratagene) was added to a final concentration of 10 ng/ μ l. This concentration of Bluescript carrier DNA (10 ng/ μ l) was included in all subsequent dilutions of samples and standards. Immediately before amplification each unknown sample was further diluted 1:10 to improve the fidelity of the PCR reaction (43). Plasmid DNAs containing bovine KL, c-kit, or IB15 subclones were used to generate standard curves from 1 ag/ μ l (10^{-15} g/ μ l) to 10 pg/ μ l (10×10^{-9} g/ μ l) each containing 10 ng/ μ l Bluescript carrier DNA. Identical 10- μ l aliquots of each sample and standard were pipetted in duplicate into a 96-well reaction plate (Marsh Biomedical Products, Rochester, NY) and sealed with adhesive film (Marsh Biomedical Products) for PCR amplification. By this design, it was possible to simultaneously assay 5 known standard concentrations and 40 unknown samples for each gene. Amplification was performed in a Perkin Elmer 9600 equipped with a heated lid using the following conditions: 0.4 μ M each primer, 16 μ M dNTPs, and 1.25 U AmpliTaq polymerase in 50 μ l GeneAmp PCR buffer (containing 1.5 mM MgCl₂) (Perkin Elmer). Each PCR amplification consisted of an initial denaturing reaction (5 min, 95 C); 25–31 cycles of denaturing (30 sec, 95 C), annealing (1 min, 60 C), and elongation (2 min, 72 C) reactions; and a final elongation reaction (10 min, 72 C). At least 0.25 μ Ci ³²P-labeled deoxycytidine triphosphate (Redivue, Amersham Life Sciences, Arlington Heights, IL) was included in each sample during amplification for detection purposes. Specific PCR products were quantitated by electrophoresing all samples on 4–5% polyacrylamide gels, simultaneously exposing the gels to a phosphor screen for 8–24 h, followed by quantitating the specific bands on a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Each gene was assayed in separate PCR reactions from the same RT samples. Equivalent steady state

mRNA levels for each gene were determined by comparing each sample to the appropriate standard curve. All KL and c-kit data were normalized for IB15.

The primers used in this quantitative amplification of KL, c-kit, and IB15 were the same as described above except for a single KL primer. A smaller 3'-primer was used for KL that facilitates the proper melting and annealing of the primer during RT: 5'-AGG CCC CAA AAG CAA ACC CGA TCA CAA GAG-3' (3' primer, 30-mer). When combined with the above 5'-KL primer, this primer amplifies both the soluble (KL1) and membrane-bound (KL2) forms of KL. This KL primer set generated a specific 452-bp KL PCR product from bovine granulosa cells that codes for the longer, soluble form of KL (example shown in Fig. 1B). A small but detectable amount of the shorter, membrane-bound form of KL was also expressed by granulosa cells (368-bp PCR product, Fig. 1B). Optimal cycle number for amplification was determined for each assay to achieve maximum sensitivity while maintaining linearity (i.e. logarithmic phase of PCR reactions). Both KL and c-kit quantitative PCR products were amplified for 31 cycles, while the IB15 PCR products were amplified for 25 cycles. The sensitivity of each quantitative PCR assay was below 1 fg, which corresponds to less than 125 fg target mRNA/ μ g total RNA. For each assay, all samples were simultaneously measured in duplicate, resulting in intraassay variabilities of 8.9% (KL), 6.0% (c-kit), and 6.5% (IB15).

Statistical analysis

All data were analyzed by a JMP 3.1 statistical analysis program (SAS Institute Inc., Cary, NC). Effects of KL, growth factor, or hormones on [³H]thymidine incorporation into DNA (Fig. 2) or steroid production (Figs. 3 and 4) were analyzed by a one-way ANOVA. Significant differences between treated cells and control (untreated) cells were determined using the Dunnett's test, which guards against the high (E0)-size (Type I) error rate across the hypothesis tests (44). Effects of follicle size on steady state KL or c-kit mRNA levels (Figs. 5 and 6) were analyzed by a one-way ANOVA as described above. Significant differences between small, medium, and large size follicles were determined using the

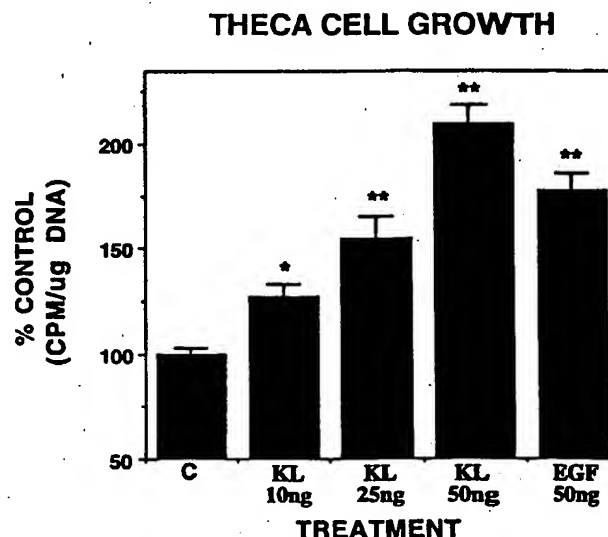


FIG. 2. Effects of KL on theca cell [³H]thymidine incorporation into DNA. Cells were cultured at subconfluent densities (growth permissive) in absence of growth factor for 24 h, then cells were treated as indicated for 20 h followed by a 4-h incubation with [³H]thymidine. Cpm of [³H]thymidine incorporated into DNA was determined and normalized to total DNA per well. Values were generally greater than 2×10^3 cpm/ μ g DNA. Data are presented as mean \pm SEM from four different experiments, each run in triplicate, and are expressed as percent of control (nontreated cells). An ANOVA was performed, and significant differences from control (untreated) cells were determined using Dunnett's test: *, $\alpha < 0.05$; **, $\alpha < 0.01$. C, control untreated cells; KL, kit ligand treatment at indicated amounts per milliliter.

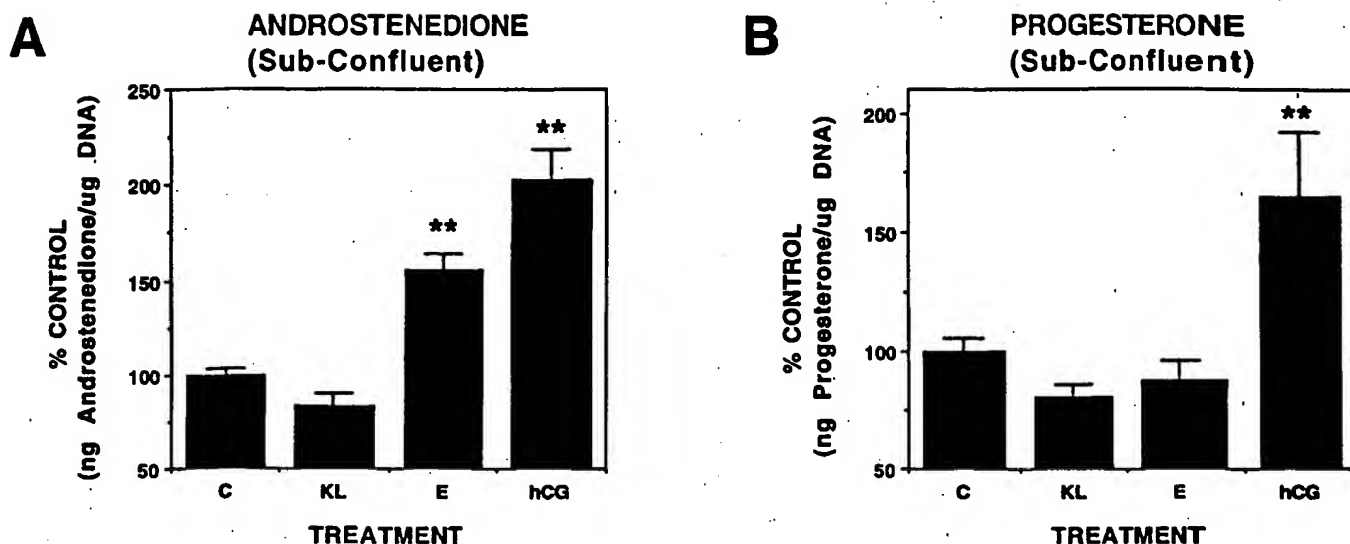


FIG. 3. Bovine theca cell steroid production at subconfluent cell densities (growth permissive). Androstenedione (A) and progesterone (B) accumulation in culture medium was determined during days 0–3 of culture. Cells were cultured in serum-free media in absence (C, control) or presence of kit ligand (KL, 50 ng/ml), estradiol (E, 10^{-7} M), or hCG (100 ng/ml). Data are presented as mean \pm SEM from nine different experiments, each run in triplicate, and are expressed as percent of control (nontreated cells). Levels of steroid accumulation in media of untreated (control) cells ranged from 50–500 ng/ μ g DNA for androstenedione and 25–400 ng/ μ g DNA for progesterone. Values with asterisks are different ($\alpha < 0.01$) from control as determined by Dunnett's test.

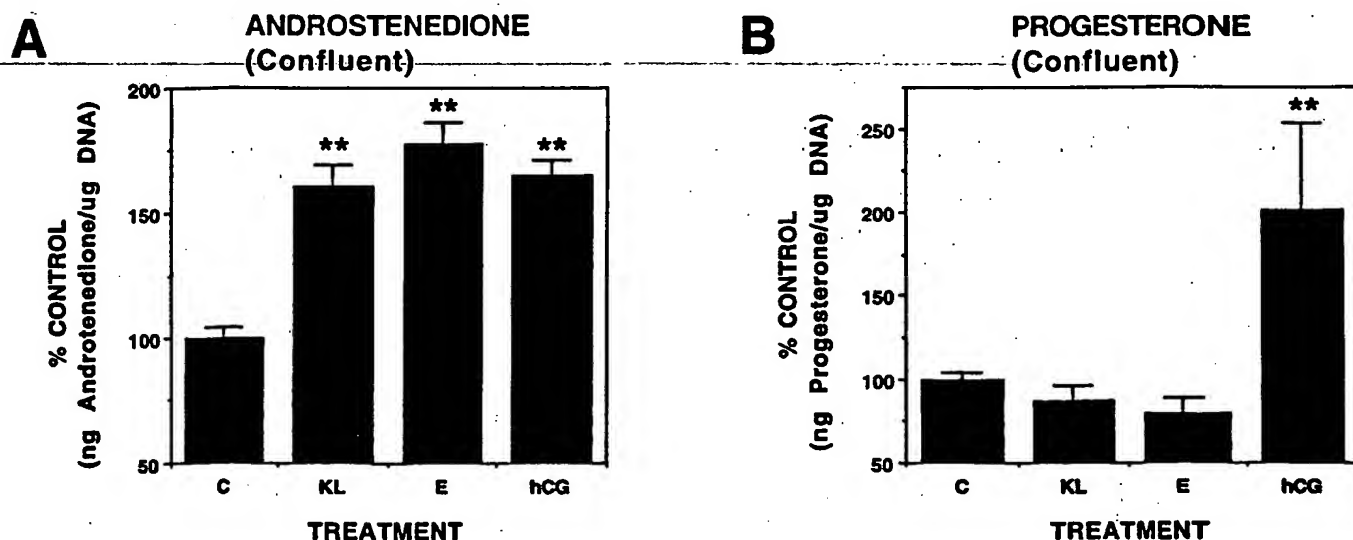


FIG. 4. Bovine theca cell steroid production at confluent cell densities (nongrowth permissive). Androstenedione (A) and progesterone (B) accumulation in culture medium was determined during days 0–3 of culture. Cells were cultured in serum-free media in absence (C, control) or presence of kit ligand (KL, 50 ng/ml), estradiol (E, 10^{-7} M), or hCG (100 ng/ml). Data are presented as mean \pm SEM from six (A) or three (B) different experiments, each run in triplicate, and are expressed as percent of control (nontreated cells). Levels of steroid accumulation in media of untreated (control) cells ranged from 40–150 ng/ μ g DNA for androstenedione and 10–150 ng/ μ g DNA for progesterone. Values with asterisks are different ($\alpha < 0.01$) from control as determined by Dunnett's test.

Tukey-Kramer HSD (honestly significant difference) test, which protects the significance tests of all combinations of pairs (45). These multiple comparisons tests are recommended for multiple comparisons with control (Dunnett's) or multiple comparisons of all pairs (Tukey-Kramer HSD) (46).

Results

Theca cells, granulosa cells, and stromal/interstitial cells were obtained from freshly isolated bovine ovaries. Cells

were independently isolated from pools of small (<5 mm diameter), medium (5–10 mm), and large (>10 mm) size follicles. Cells were used immediately for RNA preparation or placed in cell culture. The gene expression of KL and c-kit in these cells was determined by RT-PCR. Using the appropriate primers, the 315-bp KL PCR product was observed in granulosa cell RNA isolated from small, medium, and large size follicles (Fig. 1). These KL primers are designed to spe-

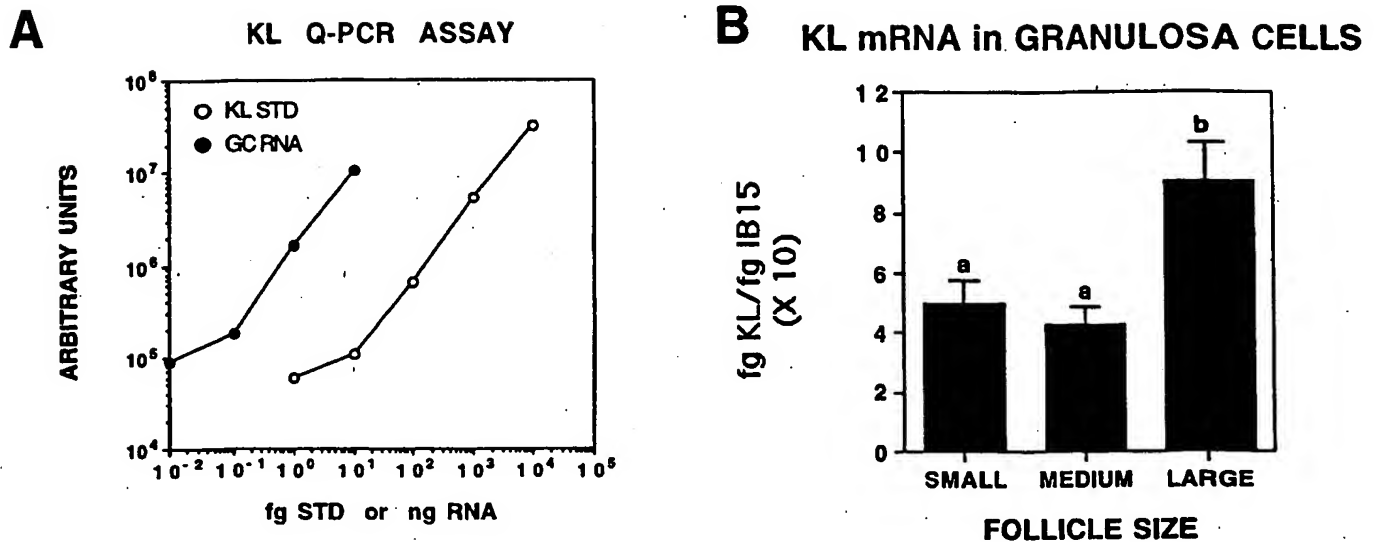


FIG. 5. KL mRNA expression by bovine granulosa cells. KL mRNA levels in granulosa cell total RNA from small, medium, and large follicles were determined using quantitative RT-PCR. A, Line graph of a typical assay validating assay. Parallel curves of a standard bovine KL subclone (○) and a fresh granulosa cell total RNA sample (●) are shown. Raw data are represented as arbitrary units \pm SEM (as read from PhosphorImager) and are directly proportional to actual cpm. B, Analysis of steady state KL mRNA levels in granulosa cells from small, medium, and large size follicles. Each sample was run in duplicate and normalized to levels of cyclophilin (IB15) mRNA. Data are presented as level of KL mRNA per IB15 mRNA, represented as mean \pm SEM from four different sets of granulosa cell RNA. An ANOVA was performed, and significant differences between follicle sizes were determined using Tukey-Kramer HSD test. Bars with different superscript letters differ from each other ($\alpha < 0.01$).

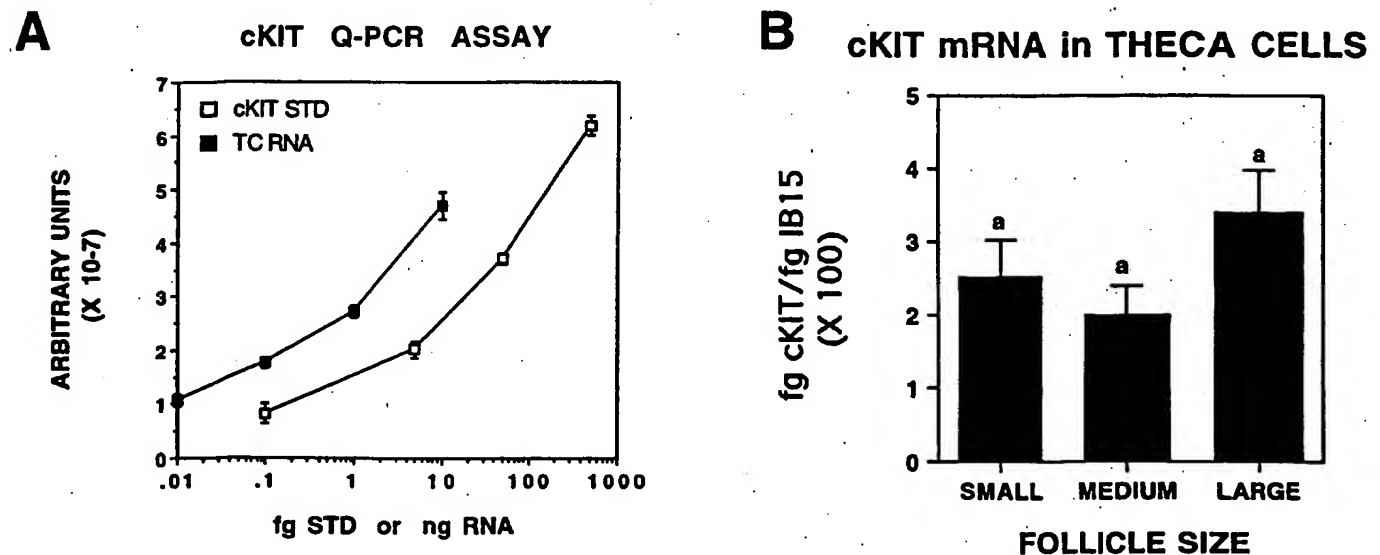


FIG. 6. c-kit mRNA expression by bovine theca cells. c-kit mRNA levels in theca cell total RNA from small, medium, and large follicles were determined using quantitative RT-PCR. A, Line graph of a typical assay. Parallel curves of a standard bovine c-kit subclone (□) and a fresh theca cell total RNA sample (■) are shown. Raw data are represented as arbitrary units \pm SEM (as read from PhosphorImager) and are directly proportional to actual cpm. B, Analysis of steady state c-kit mRNA levels in theca cells from small, medium, and large size follicles. Each sample was run in duplicate and normalized to levels of cyclophilin (IB15) mRNA. Data are presented as level of c-kit mRNA per IB15 mRNA, represented as mean \pm SEM from at least 10 different sets of theca cell RNA. No differences were seen between sizes as determined by Tukey-Kramer HSD test.

cifically amplify the soluble form of KL, which has been shown to be the predominant form expressed in rat and human ovaries (47–49). Experiments using primers designed to detect both the soluble (KL1) and membrane-bound (KL2) forms of KL confirmed that bovine granulosa cells predominantly express the soluble form of the factor (Fig. 1B). The

298-bp c-kit PCR product was observed in theca cell RNA from small, medium, and large size follicles, as well as stromal-interstitial cell RNA (Fig. 1A). These PCR products were subcloned into the Bluescript plasmid, sequenced, and confirmed to be bovine KL and c-kit cDNA (data not shown). Negative controls with the omission of RNA or reverse tran-

scriptase resulted in no PCR product being detected, demonstrating that the signal did not arise from contaminating DNA (data not shown). These results are the first to directly demonstrate receptor c-kit mRNA expression in theca cells from ovarian follicles. These results establish that bovine granulosa cells express KL and bovine theca cells express the receptor c-kit.

The ability of KL to regulate the growth of bovine theca cells was examined through an analysis of [³H]thymidine incorporation into DNA. After initial plating at subconfluent densities, freshly isolated theca cells were cultured in the absence or presence of recombinant KL for 20 h followed by [³H]thymidine for an additional 4 h. KL was found to stimulate the growth of bovine theca cells in a dose-dependent manner (Fig. 2). Preliminary studies to examine the effects of KL on theca cell proliferation used a 72-h treatment with KL followed by a DNA assay. Data suggested that KL may increase total DNA, but these results require further investigation (data not shown). EGF (50 ng/ml) was used as a positive control. These results suggest that KL is a theca cell growth factor that is at least as effective as EGF in stimulating theca cell growth. These results demonstrate that KL directly regulates theca cell growth.

To further evaluate the potential role that KL may have in regulating theca cells, the effect of KL on theca cell steroid production was examined. Initially theca cell steroid production was examined under subconfluent culture conditions (growth-permissive conditions). Under these conditions KL had no significant effect on androstenedione or progesterone accumulation in the culture medium (Fig. 3). These results are consistent with the role of KL as a theca cell growth factor that can promote entry of the cells into the cell cycle under these culture conditions. As previously demonstrated (4, 40), both estrogen (10^{-7} M) and hCG (100 ng/ml) stimulated androstenedione production, whereas only hCG stimulated progesterone production (Fig. 3). Similar experiments were performed to examine theca cell androstenedione and progesterone production at confluent densities (non-growth-permissive conditions). Theca cells do not readily enter the cell cycle under these conditions due to cellular contact inhibition. KL clearly stimulated androstenedione accumulation in the culture medium when theca cells were cultured under confluent culture conditions (Fig. 4A). The magnitude of this KL stimulation was similar to the effects of estrogen and hCG. Preliminary studies showed that the combined effects of KL and hormones were the same as either KL or hormones alone, suggesting a maximum level of stimulation was obtained (data not shown). The combined actions of KL and hormones on theca cell androgen production require further investigation. KL had no significant effect on theca cell progesterone production at any cell density (Figs. 3B and 4B). These results demonstrate that KL can directly regulate theca cell differentiation as measured by androstenedione production. These results establish KL as the only known growth factor to directly regulate theca cell steroid production in the absence of gonadotropins.

The potential regulation of KL in granulosa cells and c-kit in theca cells during follicular development was evaluated by analyzing total RNA samples from small (<5 mm), medium (5–10 mm), and large (>10 mm) size follicles. Sensitive

quantitative RT-PCR assays were developed for both bovine KL and c-kit. Under specific amplification conditions these assays used the bovine KL and c-kit PCR products shown in Fig. 1 as a template to generate standard curves. Samples consisting of total RNA from freshly isolated granulosa cells or theca cells were reverse transcribed using the specific 3' primers of the gene(s) of interest. These unknown samples were simultaneously amplified by PCR along with the known standards to quantitate gene expression. For each gene cycle, number and annealing temperature were optimized for maximum sensitivity and linearity. These quantitative assays for KL and c-kit mRNAs are extremely sensitive ($<10^{-12}$ g/sample) and have intraassay variabilities of 8.9% and 6%, respectively. As is shown in Figs. 5 and 6, each assay is linear over several orders of magnitude (0.1–1000 fg/sample). Each assay was validated by demonstrating parallel curves between the appropriate RNA samples and standards (Figs. 5A and 6A). All samples were normalized for the constitutively expressed cyclophilin mRNA (IB15) as determined by the same procedure. This normalization corrects for the amount of initial mRNA, as well as small differences in the efficiency of RT between samples. The results are shown in Figs. 5B and 6B. The steady state levels of KL mRNA in granulosa cells is higher in large size follicles than in small or medium size follicles (Fig. 5B). There are no significant differences in the steady state levels of theca cell c-kit mRNA between small, medium, and large size follicles (Fig. 6B). In addition the steady state levels of KL mRNA in granulosa cells (0.4–0.8 fg KL/fg IB15) are an order of magnitude higher than the levels of c-kit mRNA in theca cells (0.02–0.04 fg c-kit/fg IB15). These results demonstrate that the KL gene is developmentally regulated during normal follicular development and may be particularly important for theca cell function in large size follicles. The c-kit gene was shown to be constitutively expressed throughout follicular development.

Discussion

The hypothesis that was tested in this study is that KL from granulosa cells acts locally on theca cells in ovarian follicles to help regulate the mesenchymal-epithelial cell interactions between these two cell types. Many organs are composed of a functional epithelial cell type adjacent to a stromal or mesenchymal cell type. It is well established that specific mesenchymal cells produce factors that act in a paracrine manner to alter the function of adjacent epithelial cells (50–54). These cell-cell interactions control the differentiation of specific organs during development and maintain optimal cellular function in the adult. Theca-granulosa cell interactions are an example of an important mesenchymal-epithelial cell interaction in the ovary. Mesenchymal-derived theca cells have been shown to produce a number of factors including keratinocyte growth factor, hepatocyte growth factor, and transforming growth factors- α (TGF- α) and - β (TGF- β), which regulate granulosa cell function (2, 3, 55). However the role of locally produced substances from granulosa cells that feedback to regulate theca cell function has not been studied extensively. KL appears to be such a substance. Previous studies have demonstrated that granulosa cells in develop-

ing ovarian follicles express KL, which may be important for granulosa cell-oocyte interactions (5–7, 35, 36). Many of these previous studies used *in situ* and immunohistochemical techniques to demonstrate receptor c-kit expression in growing and full-grown oocytes. It was apparent in these studies that differentiated theca cells and possibly undifferentiated stromal-interstitial cells express the c-kit receptor and therefore may also respond to granulosa cell-derived KL (6, 7, 12). To examine the direct action of KL on theca cell function, it is necessary to isolate purified theca cells. The bovine ovary is large enough and available in sufficient quantities to isolate large numbers of purified theca cells (40). Also the bovine ovary is endocrinologically similar to the human and is monoovulatory. This model system is used in the current study to establish that KL can directly regulate theca cell function. KL is established as the only known granulosa cell-derived growth factor that can stimulate theca cell androstenedione production in the absence of gonadotropins. These results demonstrate that KL is an important regulator of theca cell function and may help regulate local mesenchymal-epithelial cell interactions.

Although the expression patterns of KL and c-kit in the ovary have been studied in the rat, mouse, and human, no information about the expression patterns in the bovine ovary has been published. Bovine granulosa cells from small, medium, and large size follicles were found to express the KL gene. In addition bovine granulosa cells were found to primarily express the soluble form of KL (KL1) rather than the membrane-bound form of the factor. Bovine theca cells from small, medium, and large size follicles, as well as stromal-interstitial cells, were found to express the receptor c-kit. This study is the first to directly demonstrate c-kit mRNA expression in purified theca cells. The observation that KL and c-kit mRNA's were observed in small, medium, and large size follicles demonstrates that the KL and c-kit genes are expressed throughout ovarian follicular development.

KL (also called stem cell factor, mast cell growth factor, or steel factor) can have a wide range of activities on germ cells, melanocytes, mast cells, and primitive hematopoietic cells of the myeloid, erythroid, and lymphoid cell lineages (23). Many of these multipotent stem cells alter their developmental program and differentiate in response to KL. It also appears that KL can cause many of these cell types to proliferate. Results from this study show that KL can also stimulate the growth of theca cells as measured by [³H]thymidine incorporation into DNA. The dose-response curve for KL on theca cells is similar to other cells examined (14, 15, 34, 56, 57). This observation establishes KL as the first granulosa cell-derived growth factor that can act in a paracrine manner to stimulate theca cell growth. Although IGF-I is produced in the ovary and can act on theca cells, the high circulating levels of insulin as well as the presence of IGF binding proteins in follicular fluid may limit the regulatory role of locally produced IGF-I on theca cells (58). Theca cells surround the outer layer of granulosa cells and provide the structural integrity of the follicle. The stimulation of theca cell growth by KL may be important for the formation of a thick theca interna/externa layer around healthy developing follicles. Disruption of this theca cell layer may result in abnormal follicular development. Further evaluation of the role of KL

during follicular development will require an analysis of KL actions on theca cell proliferation in small, medium, and large size follicles.

The ability of KL to regulate the cellular differentiation of several target tissues (*i.e.* mast cells, hematopoietic cells, and melanocytes) suggests that KL may also regulate the differentiated function of theca cells in the ovary. The steroidogenic capacity of theca cells is a direct reflection of functional differentiation. Therefore theca cell androstenedione and progesterone production was examined. Many growth factors stimulate DNA synthesis (*i.e.* growth) in a particular cell by promoting entry of the cell into the cell cycle (59, 60). Progression of the cell into the cell cycle results in the indirect effect of reducing the differentiated functions of the cell (61, 62). For example, TGF- α acts as a growth factor for theca cells and can reduce theca cell androstenedione and progesterone production *in vitro* (55). KL was a growth factor for theca cells under subconfluent culture conditions (*i.e.* not contact inhibited) that allow the cells to readily enter the cell cycle (growth-permissive conditions). As expected, both androstenedione and progesterone production by bovine theca cells was unaffected in response to KL. This action of KL is consistent with the ability of KL to act as a theca cell mitogen similar to TGF- α , but it does not exclude the possibility that KL can also stimulate theca cell steroid production when the cells are not growing. Therefore, theca cells were cultured under confluent conditions (*i.e.* contact inhibited) that do not allow the cells to enter the cell cycle (non-growth-permissive conditions). Interestingly KL alone stimulated androstenedione production by bovine theca cells under these conditions. Because estrogen and hCG do not directly act as growth factors for theca cells, these hormones stimulated theca cell steroid production at subconfluent and confluent cell densities. These results establish KL as the only identified growth factor made by granulosa cells that can directly stimulate theca cell androstenedione production. Granulosa cell-derived activin, inhibin, and follistatin can affect theca cell steroid production, but these factors can only augment the actions of hormones such as LH. KL stimulated androstenedione production directly. The observation that KL stimulates androstenedione but not progesterone suggests that KL may promote a follicular phase (*i.e.* high androstenedione, low progesterone production) rather than a luteal phase differentiated state of theca cells. Further evaluation of the effects of KL on theca cell differentiation during follicular development will require the isolation of theca cells from individual follicles from different stages of development.

The potential roles of KL/c-kit during normal follicular development was investigated by analyzing the regulation of KL and c-kit mRNAs in small, medium, and large size follicles. Steady state KL mRNA levels in granulosa cells were significantly higher in large size follicles than in small or medium size follicles. Steady state receptor c-kit mRNA levels in theca cells did not significantly vary among small, medium, or large size follicles. During normal follicular development, larger follicles produce increasing amounts of steroids. Eventually a single dominant follicle is selected to ovulate. The observation that KL mRNA is highest in granulosa cells from large follicles suggests that KL may be im-

portant for increased theca cell steroid production and the selection of the dominant follicle. The concentration of KL in the follicles remains to be determined. Therefore, abnormal expression/action of KL during follicle development may dramatically alter ovarian function. Overexpression of KL may result in increased numbers of developing follicles and unusually high levels of androgen production. Such events may eventually cause polycystic ovary syndrome. Abnormally low levels of KL may not be sufficient to select a dominant follicle or support later stages of follicular development. Although the current analysis of KL and c-kit used pools of small, medium, and large size follicles, the quantitative RT-PCR assays that were developed are sensitive enough to analyze KL and c-kit mRNA expression in individual follicles. Elucidation of the regulation of KL and c-kit in individual healthy, atretic, and dominant follicles may be useful in understanding the cellular mechanisms that control follicular development and dominant follicle selection.

This study shows that kit ligand is an important local regulator of ovarian follicular development. The direct actions of KL on theca cells provides new insight into the mechanisms by which KL acts in the ovary. The actions of granulosa-derived KL on theca cell growth and androstenedione production provide a feedback mechanism that may regulate mesenchymal-epithelial cell interactions in the ovary (*i.e.* theca cell-granulosa cell interactions). These cell-cell interactions may be essential for normal ovarian follicular development and reproductive function. Several mutations at the Steel locus in mice (Sl^{pan} , Sl^{con} , and Sl^l) cause ovarian follicular arrest at very early stages of follicular development. Follicular development is arrested in these mutant mice at the time that theca cells are being recruited to differentiate from the surrounding stromal cells. At least two previous studies have suggested that Sl^l mutations in mice may disrupt theca cell function (37, 38), but no direct examination of this hypothesis has been previously possible. Terada *et al.* (38) reported that suckling Sl^l mice do not produce androgens in response to LH, suggesting a theca/stromal cell malfunction. The current study helps explain why KL mutations arrest follicular development and inhibit androgen production in the ovary. The ability of KL to recruit a variety of stem cell populations to proliferate and differentiate raises the possibility that KL may also recruit undifferentiated stromal cells to differentiate into theca cells in the ovary. This recruitment of stromal cells to theca cells is an essential aspect of early primordial follicle development. This study has established the importance of KL for theca cell function during ovarian follicular development. The potential role of KL during primordial follicle development remains to be elucidated.

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Stem-cell factor in aplastic anemia: *in vitro* expression in bone marrow stroma and fibroblast cultures

Slanicka Krieger M, Nissen C, Wodnar-Filipowicz A. Stem-cell factor in aplastic anemia: *in vitro* expression in bone marrow stroma and fibroblast cultures.

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Abstract: *In vitro* expression of stem-cell factor (SCF) by bone marrow (BM) cells of 30 patients with aplastic anemia (AA) has been analyzed at the mRNA and protein levels. While no deficiencies were found in SCF mRNA expression, low levels of soluble SCF protein were measured in poorly growing AA stroma cultures. The SCF protein concentration in the supernatant and the confluence of AA stroma growth were found to correlate ($R = 0.70$). Defective proliferation was observed in the majority (20/30) of AA stroma cultures and was paralleled by poor growth of homogeneous cultures of fibroblasts from the same marrow sample. AA stroma growth was enhanced by addition of exogenous SCF in combination with interleukin-11 (IL-11), leukemia inhibitory factor (LIF), and basic fibroblast growth factor (bFGF). Our results demonstrate that deficient growth of stroma cells results in decreased production of SCF. Therefore, SCF and other stroma-derived cytokines may be of therapeutic value in AA patients with documented defects within the BM microenvironment.

M. Slanicka Krieger, C. Nissen and
A. Wodnar-Filipowicz

Department of Research and the Clinical
Department of Hematology, University Hospital
Basel, Switzerland

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SCF protein - aplastic anemia - bone marrow
cultures

Correspondence: Dr. Aleksandra
Wodnar-Filipowicz, Research Department,
University Hospital Basel, Habelstrasse 20,
4031-Basel, Switzerland

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Introduction

The pathogenic mechanisms accounting for bone marrow failure in aplastic anemia (AA) are most probably heterogeneous. Deficient or defective hematopoietic stem cells are considered to be the primary cause of the disease, but the nature of the defect(s) remains unknown (1, 2). Abnormalities intrinsic to stem cells may be accompanied by dysfunction of the BM microenvironment. Suppression of hematopoietic maturation by excessive production of growth-inhibitory lymphokines has been postulated (3, 4). Alternatively, failure of stem-cell growth may be due to the lack of, or insufficient support by, microenvironmental cells. The regulatory role of the BM microenvironment in hematopoiesis is exerted, at least in part, by production of several cytokines secreted constitutively or following stimulation (5). A stromal culture system consisting of adherent marrow fibroblasts, endothelial cells, adipocytes and hematopoietic macrophages capable of long-term *in vitro* maintenance of hematopoietic progenitor cells, is available as an *in vitro* model of the BM microenvironment (6, 7). Several investigators have used this culture system to evaluate the func-

tion of stromal cells in AA in terms of growth and hematopoietic activity. Results have shown abnormalities in function and morphology of cells in AA cultures, but the incidence of stromal defects varied widely between individual studies (8-15). The role of stroma-derived growth factors in the etiology and clinical manifestation of AA remains therefore poorly understood.

Evidence for a role of stroma-mediated defects in hematopoietic failure comes from studies on genetically anemic strains of *Sl* (Steel) mice (16). Their developmental defects are caused by mutations at the *Sl* gene locus encoding SCF, a hematopoietic cytokine produced by marrow microenvironmental cells (17, 18). SCF interacts with *c-kit*, a transmembrane receptor with tyrosine kinase activity expressed on the surface of hematopoietic stem cells; mice carrying mutations at the *W* (white spotting) *c-kit* locus display an anemic phenotype analogous to *Sl* mice (19). SCF exists in two biologically active forms: soluble and membrane-bound. The membrane-associated form is considered to play an important role in cell-cell interactions between stroma and hematopoietic stem cells within the BM microenvironment (20). Soluble SCF is a potent

stimulatory growth factor for early hematopoietic progenitor cells *in vitro* and *in vivo* (21, 22). In synergy with other growth factors, it markedly improves formation of hematopoietic colonies by precursor cells from patients with hypoproliferative marrow disorders, Diamond-Blackfan anemia (23) and aplastic anemia (24, 25). A partial *in vitro* correction of the proliferative defect of aplastic cells with SCF suggests an inadequate *in vivo* supply of this factor in AA. Our recent finding that the concentration of SCF in serum of AA patients is frequently abnormally low (26) supports this possibility.

In order to explore further the role of SCF in the pathophysiology of AA, we have analyzed *in vitro* expression of SCF mRNA and protein in cultures derived from bone marrow samples of 30 AA patients. Two types of cultures were set up: stroma cultures, containing diverse cellular components of the hematopoietic microenvironment and fibroblast cultures, representing the SCF-producing marrow stroma component (27). We have also investigated

expression of mRNA encoding the SCF receptor, *c-kit*, and characterized the effect of SCF and other stroma-derived hematopoietic growth factors, IL-11, LIF and bFGF, on growth of AA stroma cells.

Material and methods

Patients

Thirty patients with idiopathic acquired AA were included in the study (see Table 1). Three patients had not been treated previously. Twenty-seven patients had been treated with horse anti-lymphocyte globulin (ALG; Lymphoser Berna, Berne, Switzerland) in combination with high-dose corticosteroids according to a standard protocol (28). Before treatment, all patients were dependent on red blood cell and platelet transfusions and the majority of them fulfilled the criteria for severe AA (29); 3 patients had a hypoplastic marrow and severe thrombocytopenia, but were not severely granulocytopenic at presentation.

Table 1. Patient characteristics

Patient				At time of study				Therapy	Time since therapy	Current clinical condition	Current medication
No.	UPN	Age/sex [yr]	Diagnosis at presentation	Neutrophils [$\times 10^9/L$]	Reticulocytes $\times 10^9/L$	Platelets [$\times 10^9/L$]	Hb [g%]				
1	128	26/F	SAA	2.3	32	152	12.3	ALGX4	11 yr	R	CyA
2	410	31/M	SAA	1.0	68	179	12.3	ALG	2 yr	PNHlab	CyA
3	263	26/F	SAA	4.8	85	187	12.4	ALGX2	7 yr	R	None
4	291	61/F	SAA	2.9	53	290	13.9	ALG	6 yr	R	None
5	367	36/F	SAA	1.0	71	46	11.0	ALG	3 yr	R	CyA, A
6		15/F	SAA	3.2	64	148	9.2	ALG	2 yr	R	CyA, G-CSF
7	198	44/M	SAA	3.5	73	256	14.8	ALG	9 yr	R	None
8	309	34/F	SAA	1.5	62	218	12.6	ALGX3	5 yr	R	CyA, A
9	468	67/F	SAA	0.2	11	33	7.4	None*			
10	88	35/M	SAA	2.4	57	128	12.9	ALG	12 yr	R	None
11	356	5/M	SAA	1.4	56	160	12.4	ALG	4 yr	R	None
12	286	21/F	SAA	3.6	86	202	13.9	ALG	6 yr	R	None
13	236	58/M	SAA	3.8	87	145	14.8	ALG	8 yr	R	None
14	232	58/M	SAA	2.7	140	231	12.9	ALG	8 yr	R	None
15	183	23/M	SAA	1.7	85	157	13.3	ALGX4	9 yr	R	CyA
16	94	19/F	SAA	1.6	32	180	14.1	ALG	12 yr	R	None
17	352	75/F	SAA	3.0	56	220	13.4	ALG	4 yr	R	None
18	202	21/M	SAA	1.8	41	41	8.8	ALGX2	9 yr	R	CyA, A, Epo
19	197	52/M	SAA	2.5	57	141	13.5	ALG	9 yr	R	None
20	364	31/F	SAA	3.0	95	67	15.2	ALG	3 yr	R	Epo
21		20/M	SAA	0.3	26	14	8.7	None*			
22		20/F	NS	1.0	67	30	9.8	ALG	2 yr	Rp	CyA, A
23	191	14/M	SAA	26.6	53	27	7.9	ALGX2	9 yr	Rp	CyA, A
24	350	61/F	SAA	3.6	87	162	11.3	ALG	4 yr	R	CyA
25	258	22/F	NS	1.6	70	24	6.1	ALGX2	7 yr	PNH, Tx	CyA, Epo, G-CSF
26	374	43/F	NS	2.1	72	130	12.0	ALG	3 yr	R	CyA
27	467	21/F	SAA	0.4	13	20	9.8	None*			
28	249	59/M	SAA	1.9	60	236	14.1	ALG	7 yr	R	None
29	67	39/M	SAA	1.6	97	266	12.9	ALG	13 yr	PNH	CyA, A
30	408	29/M	SAA	1.8	127	80	12.6	ALG	2 yr	R	CyA, A

Abbreviations: SAA, severe aplastic anemia; NS, nonsevere aplastic anemia; *, before therapy; ALG, antilymphocyte globulin; R, remission; Rp, remission with pancytopenia; PNH, paroxysmal nocturnal hemoglobinuria; PNHlab, PNH detected only in laboratory tests; PNH, Tx, PNH with pancytopenia and transfusion dependence; CyA, cyclosporin A; A, androgens; G-CSF, granulocyte colony-stimulating factor; Epo, erythropoietin; UPN, unique patient number; patients with no UPN number assigned were treated at other institutions.

Bone marrow (BM) samples and hematopoietic colony formation assay

BM from patients and normal donors was used with informed consent. Mononuclear cells were isolated from heparinized BM by Ficoll-Hypaque density gradient centrifugation ($d = 1.077$) and BM plasma was collected for use in stroma cultures. All cultures were initiated on the day of BM aspiration. Hematopoietic colony assays were performed in 1% methylcellulose cultures containing 2×10^5 cells/ml, as described (24). Hematopoietic colonies were counted after 14 days.

Stroma cultures

Cultures were established according to Gartner and Kaplan (7) with slight modifications (11). BM mononuclear cells were seeded at 7.5×10^5 cells/ml in 8.3 cm^2 slide-flasks (Nunc, Kamstrup, Denmark) and, in parallel, in 25 cm^2 tissue culture flasks. Cells were cultured in Iscove's modified Dulbecco's medium (IMDM; Gibco, Gaithersburg, MD) supplemented with 10% fetal calf serum (FCS; *ibid*), 10% horse serum (*ibid*), 10^{-7} mol/l Dexamethasone (Roussel Uclaf, Paris, France) and 10% autologous BM plasma. After 2 weeks at 37°C , the bottoms of the slide-flasks were stained with May-Grünwald-Giemsa and growth evaluated as percentage of culture dish area covered by adherent cells. For RNA isolation, cultures in 25 cm^2 flasks were grown for 3 wk replacing half of the medium weekly. In experiments testing the effect of hematopoietic factors on stroma growth (see Fig. 5), autologous BM plasma was omitted and the following recombinant human growth factors were added: SCF at 50 ng/ml (AMGEN, Thousand Oaks, CA), IL-11 at 50 ng/ml (Genetics Institute, Cambridge, MA), LIF at 20 ng/ml (courtesy of Dr. Laura Grey, Dept. of Biochemistry, University of Oxford, Oxford, England), and bFGF at 2 ng/ml (Promega, Madison, WI or Syngene, Boulder, CO).

Fibroblast cultures

BM mononuclear cells were depleted of nonadherent cells by an overnight incubation of 1×10^7 cells in IMDM with 20% FCS at 37°C . Adherent cultures were supplemented with 2 ng/ml bFGF and fed weekly by replacing one half of the medium. The 1st day of reaching confluence was recorded as a relative measure of cell growth. For RNA isolation, fibroblasts were passaged and maintained in culture for 4 to 6 wk. Fibroblasts were characterized by immunochemical or histochemical staining and found uniformly positive for vimentin, collagens type I & III and fibronectin, and negative for CD14 and CD11c surface markers.

Determination of SCF protein concentration

Slide-flask stroma cultures were established as described above, except that autologous BM plasma was replaced by FCS to avoid addition of exogenous human SCF. After 2 wk (without medium change), supernatants were collected, depleted of nonadherent cells and stored at -70°C until assayed. SCF protein concentration was measured in duplicates using an enzyme-linked immunosorbent assay (ELISA), as described (26, 30). The standard curve showed linearity from 0.1 up to 2.5 ng SCF/ml.

Northern and reverse transcriptase-PCR (RT-PCR) analysis

RNA was isolated (31), separated in 1.1% agarose gels and transferred to Hybond membranes (Amersham, Buckinghamshire, England). The 0.9 kb HindIII-BamHI fragment of human SCF cDNA (32) was labeled with (α - ^{32}P) CTP by random priming (33) and hybridizations were performed at 42°C in a buffer containing 50% formamide. After washing at 65°C in 0.1% SSC, 0.1% SDS, blots were exposed to x-ray films for 4 d (SCF) and 2 hours (actin). For RT-PCR analysis, cDNA was prepared from 1 μg of total RNA. Oligo-dT was used as a primer for SCF and β -actin, and an antisense downstream primer for *c-kit* (5'-GATTCTGCTCAGACATCGTCG-3', pos. 2940-2960) (34). An equivalent of 200 ng of reverse transcribed RNA and 0.15 $\mu\text{mol/l}$ of each oligonucleotide primer were used in PCR reactions. In every assay, a control reaction containing water instead of RNA was carried out. The following programs were used: SCF: denature 94°C , 45 sec; anneal 60°C , 45 sec; extend 72°C , 1 min 30 sec; 40 cycles; *c-kit*: 94°C , 1 min; 60°C , 1 min 30 sec; 72°C , 1 min 30 sec; 40 cycles; β -actin: 94°C , 40 sec; 62°C , 40 sec; 72°C , 1 min; 35 cycles. The following oligonucleotide primers were used: SCF (32): sense 5'-GATGGTAGTAC-AATTGTCAGAC-3', pos. 416-437, antisense 5'-GAAGCAAACATGAAGTGTACC-3', pos. 1063-1084; *c-kit* (34): sense 5'-CGATGTGGGCAAGACTTCTGC-3', pos. 1506-1526, antisense 5'-GCCTTGGTTGGGACAACATAAG-3', pos. 2207-2228; β -actin (35): sense 5'-GAGACCTTCAACACCCAGCC-3', pos. 2038-2058, antisense 5'-CAGGAAGGAAGGCTGGAAGA-3', pos. 2447-2466. Amplified products were separated in 1.5% agarose gels and stained with ethidium bromide. For analysis of SCF mRNA, PCR products were transferred to Hybond membranes and hybridized with an internal oligonucleotide probe (5'-GATCCATTGATGCCTTCAAGGAC-3', pos. 637-659) labeled with (γ - ^{32}P) ATP using T4 polynucleotide kinase (Boehringer, Mannheim, Germany).

Results

Growth of bone marrow stroma and fibroblast cultures

Thirty patients (Table 1) and 10 normal controls were included in the study. For each patient, stroma and fibroblast cultures were initiated from the same bone marrow sample. Stroma cultures were supplemented with autologous bone marrow plasma which has been found beneficial for the formation of adherent cell layers (11 & our unpublished observations); the confluence of adherent cell foci was evaluated after 2 wk. In fibroblast cultures, the time required to reach confluence was used as a measure of cell growth. The results are summarized in Table 2, with patient numbers arranged according to increasing stroma confluence and corresponding to patient numbers presented in Table 1.

The formation of an adherent stromal layer was reduced in the majority of aplastic samples compared to controls, which all grew to 75–100% confluence. Growth impairment ranged from massive (<5–10% of normal; patients 1–6) to moderate (25–50% of normal; patients 7–20). Stroma confluence was comparable to normal in only one-third of AA marrows (patients 21–30). The differences between AA and control cells were confirmed in cultures of BM fibroblasts. Seven AA marrows (patients 1 and 4–9), which grew poorly in stroma cultures, did not generate confluent fibroblast layers within 6 wk and several others required a longer time to reach confluence than control marrows. Pulse-labeling with ^3H -thymidine confirmed these differences: incorporation into AA fibroblasts was at 1–50% of control level (not shown). In addition, morphological abnormalities were frequently observed in poorly growing AA cultures. They included missing "cobblestones areas", reduced fat cell numbers, and abnormal monocyte clusters in stroma cultures, or large, irregularly-shaped fibroblasts, often piling up in foci to give cultures a transformed appearance.

Poor cell growth was not necessarily associated with the onset of the disease. Among 3 patients analyzed before treatment with ALG, growth of stroma and fibroblast cells was severely reduced in the case of patient 9, but it was in the normal range for patients 21 and 27. The proliferative defect of BM from

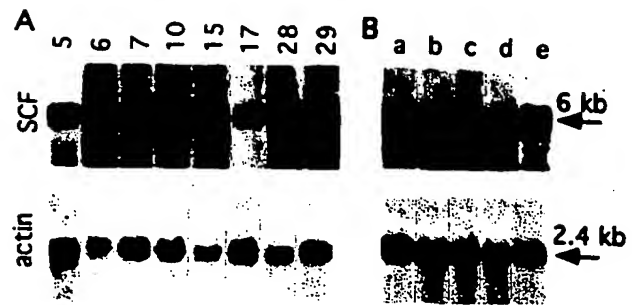


Fig. 1. Northern analysis of SCF mRNA in aplastic (A) and normal (B) cultures. Patient samples are indicated in numbers corresponding to Tables 1 & 2; normal controls are indicated by letters a–e. RNA isolated from stroma cultures was analyzed for expression of SCF and, as a reference, β -actin mRNA.

untreated and treated AA patients was more severe and more general when analyzed by the hematopoietic colony assay (Table 2). The number of colonies formed by 30 AA marrows varied between 0 to 74 per 2×10^5 cells, which was far below normal (188 ± 46).

SCF expression in stroma and fibroblast cells

RNA prepared from several stroma cultures representing poorly and well growing BM was analyzed for expression of SCF mRNA. Northern analysis showed that, irrespective of growth characteristics, cells from aplastic cultures constitutively expressed SCF mRNA in amounts comparable to control cultures (Fig. 1). RT-PCR analysis was employed in order to resolve two specific mRNA transcripts encoding soluble and membrane-bound SCF. The two alternatively spliced transcripts differ by the 84 nucleotide-long exon 6 (32) and can be visualized in one amplification reaction using oligonucleotide primers located upstream and downstream of exon 6. Both SCF mRNA species were detected in all AA and control stroma and fibroblast cultures (Fig. 2A & B). In one case (patient 4), we cannot explain the weak intensity of mRNA signals found in stroma but not in corresponding fibroblast cells. In all cultures, transcripts encoding soluble SCF were distinctly more abundant than transcripts of membrane-bound SCF. A signal corresponding to the latter form ap-

Table 2. Growth properties of patients' bone marrow cultured *in vitro*

Patient (No.)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	Control
Stroma confluence (%)	<5	<5	5	10	10	10	25	25	25	25	25	25	25	25	25	25	50	50	50	50	75	75	75	75	75	75	100	100	100	100	90 \pm 13
Fibroblast confluence (day)	>42	29	28	>42	>42	>42	>42	>42	>42	17	17	19	21	21	21	21	21	21	23	26	11	13	16	21	22	28	13	13	26	27	16 \pm 3
Hematopoietic colonies (No.)	13	3	68	4	23	21	12	16	0	8	21	24	29	37	46	74	18	23	28	17	0	31	8	36	8	16	0	34	46	49	188 \pm 46

Patient numbers correspond to numbers in Table 1; stroma confluence was determined after 14 days of culture; fibroblast growth was determined according to the 1st day of reaching confluence; hematopoietic colonies were grown in methylcellulose for 14 days (see Methods); for normal controls ($n=10$), mean \pm SD are given.

peared stronger in poorly than in well growing aplastic cultures, possibly due to an excess of macrophages which express the two mRNA forms in a 1:1 ratio (our unpublished results).

Expression of SCF was also examined at the protein level using ELISA to measure the concentration of soluble SCF released into stroma culture supernatants. Levels of SCF varied from an extremely low concentration of less than 0.1 ng/ml in very poorly growing AA cultures, to 0.7–1.0 ng/ml in well growing cultures (Fig. 3). Production of SCF reflected the proliferative potential of AA cells since a significant correlation between the concentration of SCF in the culture supernatant and the confluence of stroma growth was found ($R = 0.70$). A control culture of 75% confluence, analyzed for comparison, contained SCF at 0.52 ng/ml (not shown).

Expression of *c-kit* mRNA by stroma cultures

Aplastic stroma cells were analyzed by RT-PCR for the expression of mRNA encoding *c-kit*, the receptor for SCF (Fig. 4). A specific fragment corresponding to the transmembrane and part of the kinase domain of *c-kit* mRNA was detected in all cultures. No apparent differences between controls and patients and between poorly and well growing aplastic cultures were observed.

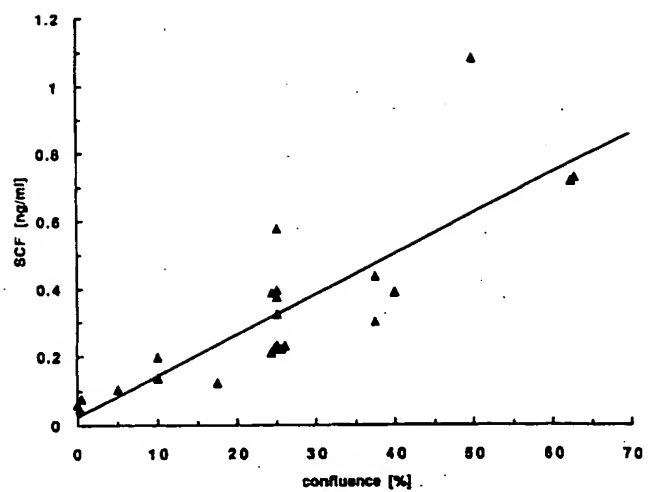
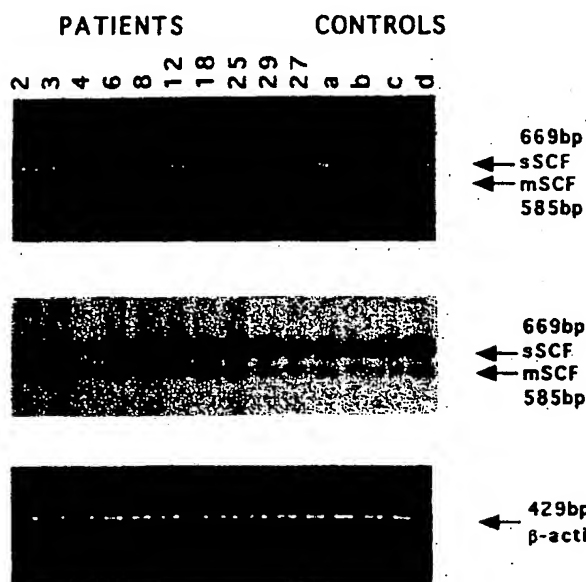


Fig. 3. Concentration of SCF protein in stroma conditioned media. Stroma cultures were established from BM samples of 23 AA patients. Conditioned media were collected and SCF levels determined by ELISA (see Methods).

Effect of hematopoietic factors on growth of stroma cultures

To examine whether growth of AA marrow microenvironmental cells can be improved by stroma-derived cytokines, we supplemented stroma cultures with SCF in combination with IL-11, LIF and bFGF. While no effect on the number and hematopoietic

(A) Stroma



(B) Fibroblasts

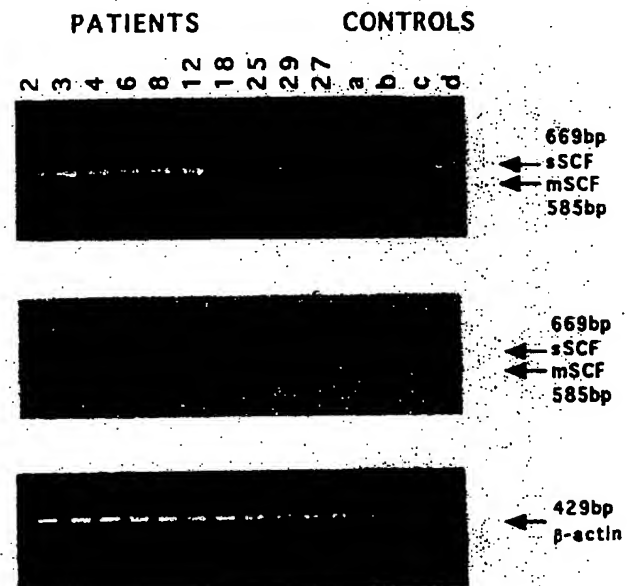


Fig. 2. RT-PCR analysis of SCF mRNA in stroma and fibroblast cultures. Patient samples are indicated in numbers corresponding to Tables 1 & 2; normal controls are indicated by letters a–d. RNA was isolated from stroma (A) and fibroblast (B) cells, reverse transcribed and amplified using SCF-specific oligonucleotide primers (see Methods). PCR products were separated in 1.5% agarose and visualized by ethidium bromide staining (upper panel) or hybridization with an SCF-specific probe following blotting (middle panel). Arrows indicate the expected size of fragments corresponding to mRNAs encoding soluble SCF (sSCF) and membrane-bound SCF (mSCF) of 669 and 585 bp, respectively. PCR amplification of β -actin cDNA was used as a control (lower panel).

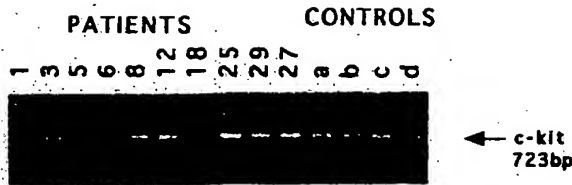


Fig. 4. RT-PCR analysis of *c-kit* mRNA in stroma cultures. Patient samples are indicated in numbers corresponding to Tables 1 & 2; normal controls are indicated by letters a-d. RNA was isolated from stroma cells, reverse transcribed and amplified using *c-kit*-specific oligonucleotide primers (see Methods). PCR products were electrophoresed in 1.5% agarose gels and visualized by ethidium bromide staining. An arrow points to the amplified 723 bp product corresponding to transmembrane and a part of kinase regions of *c-kit* cDNA.

colony-forming ability of non-adherent cells (not shown) was noted, formation of the adherent cell layer was enhanced in AA cultures by approximately 50% (Fig. 5). The increase was statistically significant when confluence of the individual cultures grown with and without growth factors was compared in the paired t-test ($p = 0.002$). SCF added alone was not effective, but it was essential in the combination, since IL-11, LIF and bFGF by themselves had only a marginal effect ($p = 0.04$). In addition to the increased confluence, the density of growth foci in adherent cell layers was distinctly higher in cultures supplemented with SCF, IL-11, LIF and bFGF.

Discussion

Expression of SCF by AA microenvironmental cells has not been well characterized. In this study, we have determined the *in vitro* expression of SCF mRNA and protein in stroma and fibroblast cultures which have been established from marrows of 30 AA patients. We have shown that poorly growing AA cultures contain abnormally low levels of soluble SCF protein. In stroma culture supernatants analyzed by ELISA, levels of secreted SCF correlated with the confluence of cell growth. Since SCF mRNA was expressed at a normal level, the reduced accumulation of SCF was, most probably, related to a reduced cell number and not to any abnormalities in the process of mRNA translation and/or protein secretion. For measurements of the concentration of SCF protein, supernatants of stroma cultures have been purposely harvested at a fixed, early time point of 2 wk when growth differences between rapidly and slowly proliferating cultures were apparent. This contrasts with a study on IL-6, GM-CSF and G-CSF (14), in which secretion of growth factors was found to be normal, but was measured at confluency reached at very different time points by individual AA marrows. Abnormally low SCF pro-

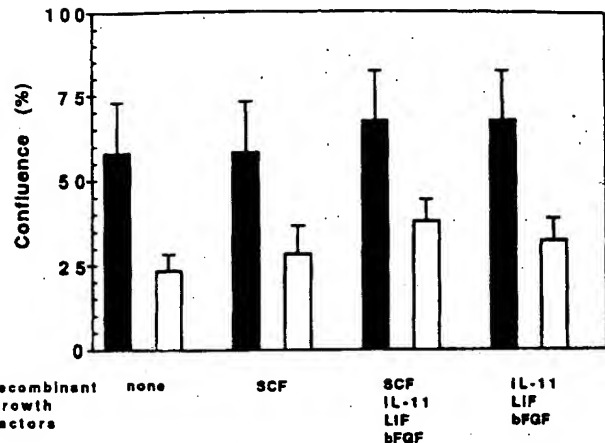


Fig. 5. Effect of recombinant growth factors on growth of stroma cultures. BM cells were cultured in the absence or presence of growth factors (for concentrations see Methods), as indicated. Confluence of adherent cell layers was determined following staining (see Methods) after 14 days. Results from 4 normal controls (shaded columns) and 14 aplastic patients (open columns) are presented. The values are mean \pm SEM.

duction *in vitro* reported in this work is consistent with the previously reported *in vivo* deficiency of SCF in sera of many AA patients (26).

Only 10 of 30 patient marrows, but all normal marrows, generated confluent layers of adherent stroma cells when cell growth was evaluated after 14 d in culture. This represents a higher incidence of proliferative defects than reported in studies using long-term AA stroma cultures maintained for at least 3–5 wk (13–15). Growth differences are more overt when analyzed early during the culture period (Nissen et al., submitted). Marrow samples which grew poorly in stroma cultures also generated aberrant and slowly growing fibroblast layers when cultured in the presence of bFGF, thus confirming abnormal *in vitro* proliferation properties of microenvironmental cells from some AA patients.

Poor cell growth in AA stroma cultures was not associated with defective expression of SCF mRNA. All cultures constitutively expressed SCF mRNA at normal levels; likewise, the ratios between the two SCF mRNA forms were similar. Transcripts encoding soluble SCF predominated over those for membrane-bound SCF. Normal expression of SCF mRNA reported here and in a few other recently described AA cases (36, 37) indicates that the proliferative defect of stromal cells in AA does not resemble the stromal defects in murine *Sl* and *Sl^d* mutants in which SCF deficiency is caused by deletions at the SCF gene locus (16).

mRNA coding for the SCF receptor, *c-kit*, was expressed in all stroma cultures examined, both aplastic and normal. The possibility of an association of AA with large deletions within the *c-kit* gene,

analogous to deletions in *W* or *W^{19H}* murine mutants (19), can therefore be excluded. Results of the *c-kit* mRNA expression do not as yet provide any information about the receptor activity, but a consistent and statistically significant improvement of stroma growth following addition of SCF together with IL-11, LIF and bFGF suggests that *c-kit* receptors present at the surface of AA marrow microenvironmental cells are functional. It appears that SCF, like other cytokines such as IL-11, LIF and bFGF, which are produced by stromal cells and which function in promoting the development of hematopoietic stem cells and in stimulating the formation of an adherent stroma layer (38), may act in a paracrine as well as autocrine fashion.

There is no obvious correlation between *in vitro* growth of marrow microenvironmental cells and the severity of the disease: marrows from 2 out of 3 patients with severe untreated aplasia gave rise to rapidly growing confluent stroma and fibroblast layers, while stroma growth and thus total SCF production was poor in many patients with apparently normal hematopoiesis after immunosuppressive treatment. Likewise diverse was growth of microenvironmental cells from 3 patients who showed symptoms of PNH as late complications of aplastic anemia. Although numbers of untreated and of PNH patients were too small in our study to allow conclusions of statistical significance, heterogeneity of SCF serum levels described previously in AA patients at presentation and in patients who developed PNH (26) is in keeping with current *in vitro* results.

The majority of our patients were examined after treatment with ALG. Since none of the patients suffered from relapse of aplasia at the time of study and only 2 (patients 22 and 23) were pancytopenic, our conclusions on *in vitro* stroma growth and SCF production concern AA patients who are in clinical remission. Immunosuppressive treatment is known to leave the patient with residual disease activity, despite near normal peripheral blood counts. This is evidenced by a persistent severe defect of hematopoietic colony formation *in vitro*. Impairment of colony growth was observed in all patients under study; it was more severe and more general than the abnormalities seen in stroma or fibroblast cultures, thus confirming previous results (13).

It might be argued that immunosuppressive treatment compromises stroma growth and function including production of hematopoietic cytokines. The available results, however, provide no evidence that cytokine levels in AA undergo changes following ALG therapy. Production of IL-6, GM-CSF and G-CSF by stromal cells *in vitro* did not correlate with response to ALG in a group of 16 patients (14). In our previous study on SCF serum levels in 32 patients, prior to and after therapy, we found that

SCF levels characteristic for individual patients remain largely unaltered through treatment-induced changes of hematopoietic function. In addition, we observed no effect of ALG and CyA on growth of stroma when tested *in vitro* (results not shown).

Whether proliferative defects of microenvironmental cells resulting in low soluble SCF production, which persist in AA after immunosuppressive therapy, are the cause or the result of hematopoietic stress due to prolonged bone marrow failure remains to be determined. The apparent deficiency of SCF may be overcome *in vivo* by redundant activities of other hematopoietic growth factors and be of no functional consequence. However, the better clinical status of AA patients with high circulating SCF serum levels (26), enhanced formation of hematopoietic colonies *in vitro* (24) and the improvement of stroma growth in response to SCF reported here, lend support to the postulated therapeutic value of SCF (39).

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Department of Surgery, Memorial Sloan-Kettering Cancer Center, New York, NY 10021, USA.

The tyrosine kinase receptor c-kit and its ligand [kit ligand (KL) or stem cell factor (SCF)] exert a broad range of biological activities during organogenesis and normal cell development. Recent studies have revealed that altered c-kit levels occur in a variety of malignancies and cancer cell lines. KL has also been shown to stimulate the growth of malignant cells, as well as to promote chemotaxis. We had previously reported expression of KL in stroma cells of normal human prostate. The present study was undertaken in order to analyze the patterns of expression of c-kit and KL in a well characterized set of prostatic tissues, including normal prostate (n=4), benign prostatic hyperplasia (BPH) (n=53) and adenocarcinoma (n=46) samples. The distribution of c-kit and KL proteins was studied by immunohistochemical analyses, while transcript levels were determined by in situ hybridization with specific RNA probes on a subset of the benign and malignant tissues referred above. In addition, reverse-transcriptase polymerase chain reaction (RT-PCR) was performed to determine levels of c-kit and KL expression in cultures of epithelial and stroma cells, as well as in the prostate cancer cell lines LNCaP, DU145 and PC3. c-kit protein in normal prostate was exclusively detected in mast cells by immunohistochemistry and in situ hybridization. However, c-kit transcripts, but not c-kit protein, were detected in low levels and with an heterogeneous pattern in basal epithelial cells of ducts and acini. c-kit in BPH was detected in epithelial cells in 9 of 53 (17%) specimens. c-kit protein expression in malignant epithelial cells was identified in 1 of 46 (2%) tumors. However, c-kit transcripts were detected in low levels by in situ hybridization in most of the tumors analyzed. KL protein and transcripts in normal prostate were detected in high levels in stroma cells. However, epithelial cells were unreactive for anti-KL antibody, but showed low levels of KL transcripts mainly in cells of the basal layer. Basal epithelial cells in hyperplastic glands showed KL expression in 13 of 53 (24%) specimens. KL protein in tumor cells was noted in 18 of 46 (39%) cases. c-kit transcripts were not found in normal prostate and in the 3 cancer cell lines analyzed by RT-PCR, however, it was present in cultured epithelial cells of BPH, and in cultures of stroma cells from both normal and BPH. The majority of cultured cell lines of epithelial and stromal origin displayed considerable levels of KL. In addition all prostate cell lines studied showed significant levels of KL transcripts. In summary, co-expression of c-kit and KL in a subset of BPH cases may suggest an autocrine mode of signaling. Data

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from this study reveals that altered patterns of c-kit and KL expression are associated with BPH and adenocarcinoma of prostate. It appears that KL induces mast cells proliferation and maturation and enhances their release of protease. This could explain the accumulation of mast cells at tumor sites, a phenomenon that was not observed in normal prostate or BPH samples.

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A Recombinant Ectodomain of the Receptor for the Stem Cell Factor (SCF) Retains Ligand-induced Receptor Dimerization and Antagonizes SCF-stimulated Cellular Responses*

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Sima Lev, Yosef Yarden†, and David Givol

From the Department of Chemical Immunology, Weizmann Institute of Science, Rehovot 76100, Israel

The stem cell factor (SCF) is a polypeptide ligand that is essential for the development of germ cells, hematopoietic progenitor cells, and melanocyte precursors. It binds to a tyrosine kinase membrane receptor that is encoded by the *c-kit* proto-oncogene. We have constructed an expression vector that directs the synthesis of the entire extracellular ligand-binding domain of the Kit/SCF receptor. When expressed and amplified in Chinese hamster ovary cells, a secreted 90-kDa glycoprotein could be harvested from the growth medium of the cells in a soluble form. This extracellular portion of the Kit/SCF receptor, denoted Kit-X, was recognized by antibodies specific to the SCF receptor; and when injected into animals, it raised antibodies that were reactive with the complete membrane form of the receptor. Direct binding and covalent cross-linking of radiolabeled SCF showed that Kit-X fully retained high affinity ligand binding and also underwent efficient dimerization in the presence of the ligand. The capacity of Kit-X to act as an antagonist of SCF was assayed on cultured cells that overexpress the receptor. Simultaneous addition of SCF and Kit-X to these cells resulted in a stoichiometric inhibition of SCF binding and a consequent decrease in autophosphorylation of the SCF receptor on tyrosine residues. The inhibition extended to later SCF-mediated responses, including the association of the receptor with phosphatidylinositol 3'-kinase and coupling to the Raf1 protein kinase. These results indicate that the recombinant ectodomain of the Kit-SCF receptor can be used as a specific antagonist of SCF actions and may enable detailed molecular analysis of ligand-receptor interactions.

Polypeptide growth factors are soluble mitogens that act upon their target cells after binding to specific cell-surface receptors. The recently identified stem cell factor (SCF)¹ (also called mast cell growth factor and *kit* ligand) (Huang *et al.*,

1990; Nocka *et al.*, 1990; Anderson *et al.*, 1990; Copeland *et al.*, 1990; Williams *et al.*, 1990; Martin *et al.*, 1990; Zsebo *et al.*, 1990a, 1990b; Flanagan and Leder, 1990) is an example of such a growth factor. The 31-kDa glycoprotein is produced by certain mesenchymal cells as a soluble or cell-surface glycoprotein (Flanagan *et al.*, 1991). Although little is known about the biological functions of SCF, the finding that the steel (*Sl*) locus encodes murine SCF (Copeland *et al.*, 1990; Zsebo *et al.*, 1990b; Huang *et al.*, 1990) implicates the ligand molecule in the development and migration of neural crest-derived melanocyte, germ cells, and hematopoietic progenitors (Russell, 1979; Silvers, 1979).

The biological effects of SCF are mediated by a 145-kDa cell-surface glycoprotein that is encoded by the *c-kit* proto-oncogene (Yarden *et al.*, 1987; Qiu *et al.*, 1988). The SCF receptor shows structural similarity to other growth factor receptors and especially to the receptors for the platelet-derived growth factors (PDGFs) and the macrophage growth factor (colony-stimulating factor 1) (reviewed by Yarden and Ullrich (1988)). These receptors share the same general structure, which includes a large extracellular ligand-binding region with regularly spaced cysteine residues that define five β -strand-rich immunoglobulin-like domains (Williams and Barclay, 1989). A single transmembrane stretch of amino acids connects the exoplasmic portion with a cytoplasmic tyrosine kinase domain. Mutations within this latter region were identified in various white spotting (*W*) mutant mice (Nocka *et al.*, 1990; Tan *et al.*, 1990; Reith *et al.*, 1990), which display a phenotype identical to that of *Sl* mutants. In addition, a viral gene that encodes essentially only the tyrosine kinase portion of the SCF receptor was identified as the transforming gene (*v-kit*) of the HZ4 feline sarcoma virus (Besmer *et al.*, 1986).

Signal transduction by growth factors is initiated by ligand-induced dimerization of the receptors (Yarden and Schlessinger, 1987a, 1987b; Ullrich and Schlessinger, 1990). This is followed by catalytic activation of the tyrosine kinase function, which in turn phosphorylates itself and several cytoplasmic proteins on tyrosine residues. In the case of the PDGF receptor, these include phospholipase C₂, the GTPase-activating protein, Raf1, and a presumed subunit of phosphatidylinositol 3'-kinase (reviewed by Cantley *et al.* (1991)). A slightly different set of effector proteins appears to couple to the ligand-stimulated tyrosine kinase of the Kit-SCF receptor (Lev *et al.*, 1991).

In analogy to the ligands of the Kit-related receptors for PDGF and colony-stimulating factor 1 (Heldin and Westermark 1990; Sherr, 1990), persistent stimulation of the kinase function of the SCF receptor, as part of a chimeric molecule, results in phenotypic transformation (Lev *et al.*, 1990). This suggests that SCF may be involved in malignancy or other

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† Incumbent of the Armour Family Chair for Cancer Research.

¹ The abbreviations used are: SCF, stem cell factor; PDGF, platelet-derived growth factor; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; EGF, epidermal growth factor; SDS, sodium dodecyl sulfate; WGA, wheat germ agglutinin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenedis(oxyethylene-nitrilo)] tetraacetic acid; BS³, bis(sulfosuccinimidyl)suberate.

hyperproliferative states. Interference of such SCF-mediated autocrine or paracrine loops may therefore be of clinical value. In this study, we present the construction, expression, and biological properties of a potential antagonist of SCF function, namely a soluble exoplasmic domain of the SCF receptor. Our finding that this recombinant protein retained high affinity ligand binding and receptor dimerization indicates that a dimerization site of the receptor resides in the exoplasmic portion and reinforces the feasibility of this approach for antagonism of hormone action.

MATERIALS AND METHODS

Construction of pDHFR-Kit-X Expression Vector, Transfection, and Selection of a Overexpressing CHO Cell Subline

An expression vector that directs the synthesis and secretion of the ectodomain of Kit (Kit-X) was constructed as follows. A 1560-base pair *Bam*HI-*Dra*I fragment of the human *c-kit* cDNA (Yarden *et al.*, 1987), encoding the extracellular portion of the SCF receptor, was subcloned into a Bluescript plasmid (Stratagene) that was pre-cut with *Bam*HI and *Eco*RV restriction enzymes. The resulting pBS-Kit-X was then cleaved open with *Cl*al and *Xho*I and ligated into a preannealed pair of synthetic oligonucleotides. The nucleotide sequences of the synthetic 17- and 19-base adaptors are given in Fig. 1 together with their translation outcome. This adds 5 amino acids and two in-frame stop codons to the carboxyl terminus of the ectodomain. After ligation and transformation of bacteria, the plasmid was cut with *Bam*HI and *Xba*I to yield a 1.5-kilobase DNA fragment that was inserted into a mammalian expression vector (pSV-DHFR). The latter is our modification of the previously described pLSV vector (Laub *et al.*, 1983; Livneh *et al.*, 1986). This modified plasmid was cut downstream of the SV40 promoter at *Xho*I and *Xba*I sites and ligated to the 1.5-kilobase fragment. The final configuration of the pDHFR-Kit-X expression vector is shown in Fig. 1. It controls transcription of the truncated *kit* by the SV40 early promoter and independently directs expression of the dihydrofolate reductase gene by the thymidine kinase promoter.

The pDHFR-Kit-X DNA was transfected into *dhfr*-deficient CHO cells (Urbaub and Chasin, 1980) by the calcium phosphate precipitation method, and stably expressing cells were selected by their resistance to methotrexate added to nucleoside-free medium supplemented with 10% dialyzed calf serum. The resulting colonies were screened for secretion of Kit-X by metabolic labeling with [³⁵S]methionine. For metabolic labeling, colonies grown in 24-well dishes were labeled with 50 μ Ci of [³⁵S]methionine (Amersham Corp.) in 1 ml of medium supplemented with dialyzed calf serum. Following 12 h of labeling, the medium was harvested, cleared of cell debris, and subjected to immunoprecipitation with 5 μ l of an antiserum obtained from mice injected with murine fibroblasts that overexpress human *c-kit* (antiserum TD). Immunocomplexes were adsorbed to protein A on agarose beads (Bio-Rad) and washed extensively before separation by SDS gel electrophoresis. Positive clones underwent gene amplification by sequential treatment with increasing concentrations of methotrexate up to 1 μ M. The highest secretor clone, denoted Kit-X-14, was selected for mass production of the protein.

Purification of Kit-X from Conditioned Medium

Kit-X-14 cells were grown to near confluence in 15-cm dishes. The monolayers were then washed twice with phosphate-buffered saline, and serum-free medium was added. Following 8–12 h at 37 °C, the medium was discarded and replaced with a minimal volume of fresh medium. The cells were left at 37 °C for 2–4 days before harvest. The collected conditioned medium, in batches of up to 500 ml, was cleared by centrifugation and concentrated 10-fold using an Amicon ultrafiltration cell equipped with a membrane filter with a *M*_w cutoff of 30,000. The resulting concentrate was mixed with 2–4 ml of agarose beads containing WGA (2 mg/ml packed beads). After 2 h of incubation with shaking at 4 °C, the beads were packed in a column that was washed extensively with 50 mM Tris-HCl (pH 7.5) containing 0.5 M NaCl. Elution of bound glycoproteins was achieved with 0.3 M *N*-acetylglucosamine (in 50 mM Tris-HCl (pH 7.5)). The eluate was dialyzed against PBS and analyzed by gel electrophoresis to determine purity.

Generation of Antisera to Kit-X

Lectin-purified preparations of Kit-X (100 μ g each) were used for subcutaneous injections in rabbits and mice. The first injection included complete Freund's adjuvant, and the following booster injections (at 2-week intervals) contained incomplete Freund's adjuvant. Animals were bled 10 days after the third booster, and the sera were tested by an immunoprecipitation assay. The spleens of some of these mice were used to generate hybridoma that secretes a monoclonal anti-Kit-X antibody (Kit 94).²

Radiolabeling of Kit-X and SCF

Lectin-purified Kit-X (10 μ g) was labeled by the lactoperoxidase method as follows: Kit-X (70 μ l) in PBS was mixed with 40 μ l of 0.5 M sodium acetate buffer (pH 5.6) and 1 mCi of Na¹²⁵I (Amersham Corp). Lactoperoxidase (2.5 μ g/ml, 10 μ l) was added, and the reaction was started by adding 10 μ l of dilute H₂O₂ solution (0.002% in water). After 5 min at 22 °C, the reaction was stopped by the addition of 0.5 ml of tyrosine (2 mg/ml). Radiolabeled Kit-X was separated from free iodine by gel filtration on a Sephadex G-15 column (0.8 \times 7 cm) that was presaturated with gelatin (0.2% in PBS).

Recombinant human SCF made in bacteria (Zeebo *et al.*, 1990) was labeled by the chloramine-T method (Hunter and Greenwood, 1962) and separated from free iodine by gel filtration as described above for Kit-X. The specific activity of the pooled fractions (1–2 \times 10⁶ cpm/ng) was calculated on the basis of precipitation assay with trichloroacetic acid.

¹²⁵I-SCF Binding to Kit-X

Direct Binding to Kit-X—Direct binding of SCF to Kit-X was measured by immobilization of Kit-X as follows. Goat antibodies to mouse immunoglobulin G were used to coat 96-well plates (50 μ l/well) at a concentration of 25 μ g of affinity-purified antibody (Jackson Laboratory, Ann Arbor, MI)/ml of PBS. Following 12 h of incubation with the antibodies at 4 °C, the antibody solution was replaced with a 20 μ g/ml solution of the Kit 94 mouse monoclonal antibody to the human Kit/SCF receptor. The monoclonal antibody was removed after 3 h at 22 °C, and nonspecific adsorption to the wells was blocked by 2 h of incubation with PBS containing 1% BSA. Following washing, this lectin-purified Kit-X (100 ng/well, in PBS plus 1% BSA) was added for 16 h at 4 °C. The plates were washed three times with PBS containing 1% BSA, and then different concentrations of ¹²⁵I-SCF were added. After incubation for 2 h at 22 °C, the wells were washed three times with PBS containing 1% BSA, and the radioactivity of individual wells was determined in a γ -counter. Nonspecific binding was determined by using wells that did not receive either Kit-X or the monoclonal antibody. Both resulted in <15% of the total binding signal.

Inhibition of ¹²⁵I-SCF Binding to Intact Cells—T18 cells were established by transfection of *dhfr*-deficient CHO cells with the full-length cDNA of human *c-kit* according to the protocol described above for Kit-X expression. For competitive binding assays with Kit-X, we used confluent monolayers of the T18 cells in 24-well plates. The monolayers were washed once with binding buffer (RPMI 1640 medium containing 25 mM Hepes (pH 7.5) and 0.1% BSA) and then incubated with 0.25 ml of binding buffer containing a constant amount of ¹²⁵I-SCF and various concentrations of lectin-purified Kit-X (0.02 nM to 1.5 μ M) or unlabeled SCF. After 90 min at 22 °C, the cells were washed four times with binding buffer, and their radioactivity was determined by solubilization and harvest in 0.2 N NaOH and 0.1% SDS.

Covalent Cross-linking of ¹²⁵I-SCF

WGA-purified Kit-X was incubated with ¹²⁵I-SCF at various concentrations in PBS. After 2 h at 4 °C, the reaction mixtures were transferred to 22 °C, and the proteins were cross-linked by adding bis(sulfosuccinimidyl)suberate (BS³) (Pierce Chemical Co.). Thirty min later, the reaction was terminated with 150 mM glycine HCl (pH 7.5). Protein A Sepharose coupled to the Kit 94 monoclonal antibody was added 5 min later, and Kit-X was immunoprecipitated and resolved by electrophoresis on 5.5% acrylamide gels.

In Vivo Tyrosine Phosphorylation Assay

T18 cells were grown to confluence in 6-well plates and then labeled for 4 h by incubation at 37 °C with phosphate-free Dulbecco's modi-

² S. Lev, Y. Yarden, and D. Givol, manuscript in preparation.

fied Eagle's medium supplemented with 0.1% dialyzed calf serum and 0.5 mCi of ortho[32 P]phosphate (9000 Ci/mmol; Kamag, Beersheva, Israel). At the end of the labeling period, SCF was added to a final concentration of 3.3 nM. Lectin-purified Kit-X was also added to some wells as indicated. Stimulation by SCF lasted 15 min at 22 °C and was followed by solubilization of the cells in 1 ml of lysis buffer (50 mM Hepes/NaOH (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM EGTA, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 0.2 mM sodium orthovanadate, and 100 mM sodium fluoride). Unsolubilized material was removed by centrifugation at 13,000 × g (15 min, 4 °C), and the cleared lysate was incubated with Sepharose coupled to the monoclonal antibody to phosphotyrosine (1G2) (Huhn *et al.*, 1987). Unbound proteins were removed after 45 min at 4 °C by washing with HNTG buffer (20 mM Hepes/NaOH (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, 10% glycerol). The adsorbed proteins were eluted by incubating the agarose beads with HNTG buffer containing 50 mM phenyl phosphate. Recovered proteins were incubated with Sepharose-bound antibodies directed to the carboxyl-terminal peptide of the Kit/SCF receptor (antibody 212). This was followed by boiling in gel sample buffer and SDS gel electrophoresis for detection of the tyrosine-phosphorylated Kit/SCF receptor.

Assay of Phosphatidylinositol 3'-Kinase Activity

Confluent monolayers of T18 cells (5×10^6 cells) were incubated with SCF and Kit-X at various concentrations for 10 min at 22 °C. The cells were then solubilized in lysis buffer, and the Kit/SCF receptor was immunoprecipitated with antibody 212 (anti-carboxyl-terminal peptide). Phosphatidylinositol 3'-kinase activity in the immunoprecipitates was assayed as previously described (Lev *et al.*, 1991).

Determination of Raf1 Electrophoretic Mobility Shift

Confluent monolayers of T18 cells (5×10^6 cells) were stimulated with 3.3 nM SCF at 37 °C in the presence or absence of Kit-X as indicated. The Raf1 protein was then immunoprecipitated from the stimulated cells, gel-electrophoresed, and Western-blotted with antibodies to Raf1 as has been previously described (Lev *et al.*, 1991).

RESULTS

Construction and Expression of Recombinant Exoplasmic Domain of the SCF Receptor—To generate a functional secreted SCF receptor, we used a portion of the human *kit* cDNA (Yarden *et al.*, 1987) that encodes a 507-amino acid protein, including the signal peptide and essentially the whole extracellular domain but the 13 amino acids preceding the transmembrane region. All the cysteine residues were included in this portion to retain the configuration of the immunoglobulin-like domains (Williams and Barclay, 1989). Five additional amino acids were added to the carboxyl terminus by insertion of the corresponding oligonucleotides that also encoded translation stop codons and an *Xba*I site (Fig. 1). A plasmid vector carrying the above-described DNA under the regulation of the SV40 promoter and also containing a dihydrofolate reductase (*dhfr*) gene as a selectable marker was used to transfect *dhfr*-deficient CHO mutant cells (Urlaub and Chasin, 1980). Methotrexate-resistant colonies were grown separately and selected for expression of the Kit-X protein by using an immunoprecipitation assay with an anti-serum specific to the human SCF receptor (anti-TD antibody). This analysis revealed a 90-kDa protein in the medium of transfected cells that was absent from the medium of control untransfected cells (Fig. 2, upper). The apparent molecular mass of the secreted protein was much higher than the predicted molecular mass (56 kDa), and its appearance on SDS-polyacrylamide gel electrophoresis was relatively diffuse. Both observations may be attributed to sugar residues, which contribute exactly the same mass to the full-length receptor (Yarden *et al.*, 1987). Indeed, the Kit-X protein was recognized by the wheat germ lectin (WGA) and could be specifically eluted from the lectin column with the corresponding sugar

(see below). A cell clone designated Kit-X-14, which resisted 1 µM methotrexate and secreted ~4 µg of Kit-X/ml in 24 h, was selected for mass production of the recombinant protein. Batches of 200–300 ml of conditioned medium were prepared, and the Kit-X protein was purified by WGA affinity chromatography. The *N*-acetylglucosamine-eluted fraction contained 80–90% pure Kit-X as determined by Coomassie Blue staining and radiolabeling with 125 I and lactoperoxidase (Fig. 2, lower).

As a test for structural conservation, we examined the immunogenic potential of the Kit-X protein. Lectin-purified preparations were therefore injected subcutaneously into mice and rabbits. Immunized animals were bled 10 days after the third booster injection, and antisera were tested for recognition of the native membrane form of the SCF receptor. As shown in Fig. 3, both the mouse (MX) and the rabbit (RX) antisera specifically immunoprecipitated the human SCF receptor from [35 S]methionine-labeled cells that overexpress it. The spleen of an immunized mouse was later used to generate the Kit 94-IgG₁ monoclonal antibody that was used in some of our experiments.² These results prove the structural integrity of Kit-X and demonstrate the usefulness of the recombinant protein for immunological purposes.

Binding of SCF to Soluble Kit-X Protein—Three independent biochemical assays confirmed that the recombinant ectodomain retained ligand binding capacity. These are direct binding of 125 I-SCF to immobilized Kit-X, competition of Kit-X with the radiolabeled ligand for binding to the cell-surface receptor, and covalent cross-linking of 125 I-SCF. The results of the immobilized Kit-X assay are shown in Fig. 4. The secreted protein was bound on a flexible 96-well dish by means of two layers of antibodies. The first consisted of goat antibodies to mouse IgG, and it was overlaid with a monoclonal antibody to the SCF-receptor (Kit 94-IgG₁). Nonspecific ligand binding was determined by performing the assay in the absence of Kit-X or of the Kit 94-IgG₁ monoclonal antibody. Specific and saturable binding of SCF was observed after subtraction of the nonspecifically bound ligand (<15% of total binding). Scatchard analysis of the binding data (Fig. 4) revealed the presence of a single population of binding sites with a dissociation constant of 0.7 nM. Thus, the soluble form of the SCF receptor fully retained the high affinity binding of the intact full-length receptor for SCF ($K_d = 1$ nM) (Huang *et al.*, 1990; Flanagan and Leder, 1990).³

Soluble Ectodomain Inhibits SCF Binding to the Native Receptor—Given the high affinity of Kit-X for SCF (Fig. 4), it was expected to compete with the native membrane receptor for binding of the ligand. This possibility was examined by using a CHO cell line (T18) that overexpresses the human SCF receptor as a result of transfection with the *c-kit* cDNA. Lectin-purified Kit-X was added at various concentrations to the T18 cells together with a constant concentration of 125 I-SCF. Following 90 min of incubation at 22 °C, the amount of cell-bound 125 I-SCF was determined. As depicted in Fig. 5B, the soluble ectodomain inhibited SCF binding in a concentration-dependent manner. For comparison, the same experiment was repeated with increasing concentrations of unlabeled SCF (Fig. 5A). In both experiments, 50% inhibition of the binding of SCF was obtained with $\sim 10^{-9}$ M competitor. Thus, the soluble Kit-X protein is capable of stoichiometric inhibition of SCF binding to the cell-surface receptor.

Dimerization of the Kit-X Protein in Presence of SCF—When radiolabeled recombinant SCF was incubated with a lectin-purified preparation of the Kit-X protein followed by covalent cross-linking with BS³, mostly a 220-kDa radioactive

³ S. Lev, Y. Yarden, and D. Givol, unpublished data.

FIG. 1. Construction of mammalian expression vector encoding Kit-X. A 1560-base pair DNA fragment of the human *c-kit* cDNA was subcloned into the Bluescript vector. A pair of synthetic oligonucleotides (boxed sequences) was then ligated into the 3'-end of the cDNA. In this configuration, translation of the *c-kit* cDNA yielded the indicated amino acids (in three-letter code) at the carboxyl terminus of the extracellular domain. Restriction of the truncated *c-kit* cDNA with *Bam*HI and *Xba*I endonucleases enabled ligation into the pDHFR expression vector downstream of the polyoma SV40 promoter. The plasmid also contained the dihydrofolate reductase (*DHFR*) gene driven by the thymidine kinase promoter. *kbp*, kilobase pairs.

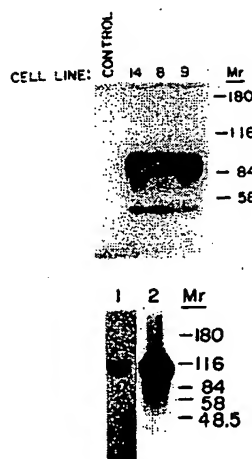
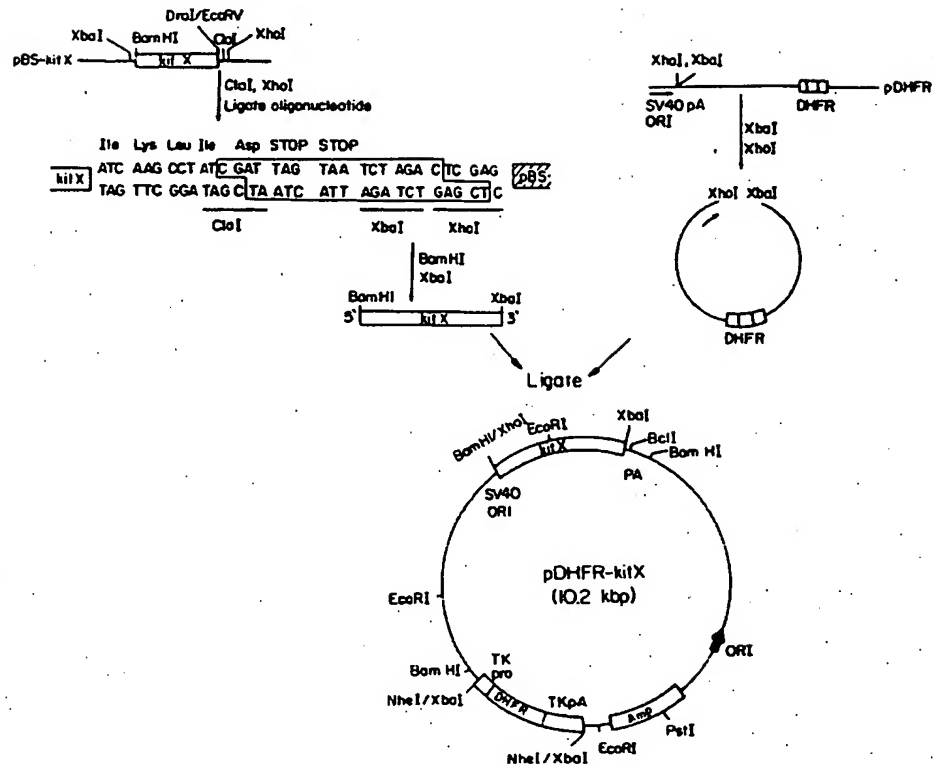


FIG. 2. Expression and purification of Kit-X protein from medium of transfected CHO cells. Upper, expression in CHO cells. Drug-resistant clones of transfected CHO cells (indicated by their numbers) or untransfected cells (CONTROL) were metabolically labeled for 12 h with [35 S]methionine. Supernatants (1 ml) of each cell line were then immunoprecipitated with a murine antiserum directed to the extracellular domain of the Kit protein (antiserum TD). Immunocomplexes were extensively washed and resolved by gel electrophoresis. An autoradiograph of the dried gel (12-h exposure to Kodak XAR film with intensifier) is shown along with the locations of molecular weight marker proteins. Lower, lectin purification of Kit-X. Serum-free supernatants of clone 14 were pooled and subjected to affinity chromatography on immobilized WGA followed by specific elution with *N*-acetylglucosamine (see "Materials and Methods"). Lane 1, results of Coomassie Blue staining of the gel-separated purified fraction; lane 2, autoradiograph of the same fraction after radiolabeling with Na^{125}I and lactoperoxidase and gel filtration on a Sephadex G-15 column.

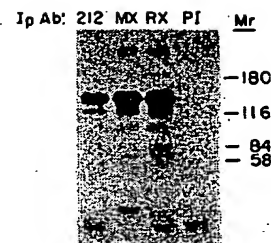


FIG. 3. Immunoprecipitation of Kit/SCF receptor with antisera raised against lectin-purified Kit-X protein. Lectin-purified preparations of Kit-X (100 μg each) were used to immunize either mice or rabbits by subcutaneous injection. Animals were bled 10 days after the third booster injection, and sera were tested by immunoprecipitation of p145^{Kit} from [35 S]methionine-labeled T18 cells (equivalent of 10^5 cells/lane). An autoradiograph (24-h exposure) of the gel-separated immunocomplexes is shown along with the locations of molecular weight marker proteins. The antisera used were as follows: 212, a rabbit antiserum against the carboxyl-terminal peptide of Kit; MX, serum from Kit-X-injected mice; RX, serum from Kit-X-injected rabbits; PI, preimmune rabbit serum. Ip Ab, immunoprecipitated antibody.

band was observed (Fig. 6, upper). A faint band of 110–120 kDa was also resolved. The appearance of both labeled bands was strictly dependent on the presence of the cross-linking reagent (Fig. 6, upper). In addition, cross-linking in the absence of SCF followed by Western blotting showed only the 90-kDa form (Fig. 6, lower). Based on the analogy between the SCF receptor and the receptors for PDGF (Bishayee *et al.*, 1989; Heldin *et al.*, 1989, 1990; Duan *et al.*, 1991) and EGF (Cochet *et al.*, 1988), we assume that the lower molecular mass band represents monomeric Kit-X cross-linked to radiolabeled recombinant SCF (18 kDa), whereas the higher molecular mass species is due to a dimer of the soluble ectodomain that binds one or two molecules of the ligand. The

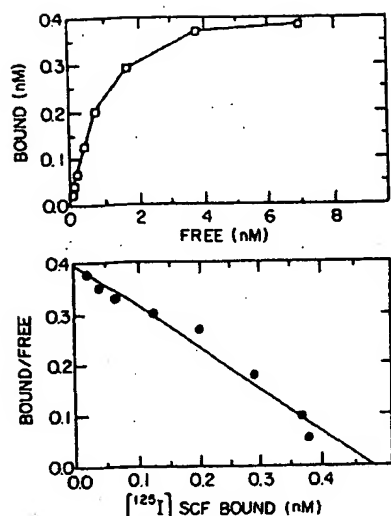


FIG. 4. Binding of ¹²⁵I-SCF to immobilized Kit-X. 96-Well microtiter plates were coated with 1 μ g of monoclonal antibody to Kit (Kit 94-IgG₁) and then with Kit-X (2 μ g/ml of solution in PBS containing 1% bovine serum albumin). The plates were then washed extensively with PBS containing 1% BSA and incubated with ¹²⁵I-SCF at various concentrations. Following 2 h at 22 °C, the plates were extensively washed, and the radioactivity of each well was determined in a γ -counter. Nonspecific binding of SCF was determined as described under "Materials and Methods." The results given are averages of duplicate determinations and are described either directly as bound SCF versus free ligand (upper) or as a Scatchard plot (lower).

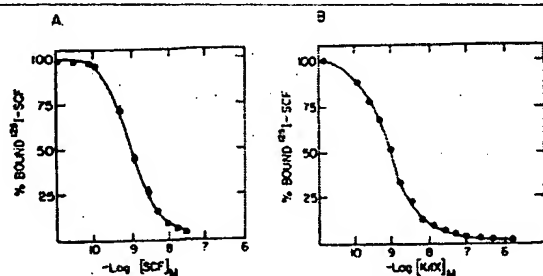


FIG. 5. Competition of Kit-X with ¹²⁵I-SCF for binding to T18 cells. Monolayers of T18 cells, which overexpress the human Kit protein, were grown in 24-well plates and assayed at confluence for binding of radiolabeled SCF (0.16 nM). The binding reaction was performed as described under "Materials and Methods" in the presence of either unlabeled SCF (0.02–30 nM) (A) or the lectin-purified Kit-X protein (0.02 nM to 1.5 μ M) (B). The results given are averages of duplicate determinations (bars that represent the ranges are shown when large enough to be visible), and are expressed as the extent of inhibition of the specific binding in the absence of competitor (taken as 100%).

specificity and saturability of the dimerization reaction were examined by performing the chemical cross-linking reaction in the presence of increasing concentrations of ¹²⁵I-SCF (Fig. 6, center, left panel), unlabeled Kit-X (middle panel), or unlabeled SCF as a competitor (right panel). Besides demonstrating the specificity for SCF, this analysis showed that the quantitative relationships between the monomeric and dimeric forms of the truncated receptor were not significantly affected when the concentrations of the ligand or the soluble receptor varied by 2 orders of magnitude; the dimeric form predominated under all conditions.

Based on these results, we concluded that the exoplasmic domain of the SCF receptor is sufficient for dimer formation, unlike the case with the epidermal growth factor receptor

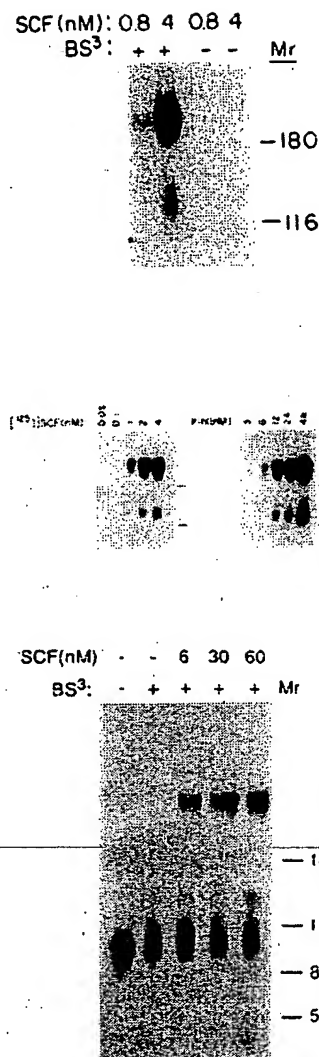


FIG. 6. Ligand-induced dimerization of Kit-X. Upper, lectin-purified Kit-X (50 nM) was mixed with ¹²⁵I-SCF (0.8 or 4 nM) in a total reaction volume of 0.05 ml. The chemical cross-linker BS³ was added where indicated and incubated for 30 min at 22 °C. The reaction was stopped by the addition of 50 mM glycine HCl (pH 7.5), and the cross-linked complexes were immunoprecipitated with the Kit 94-IgG₁ monoclonal antibody. The washed immunocomplexes were resolved on 5.5% polyacrylamide gel. An autoradiograph (2-h exposure) is given along with the locations of molecular weight marker proteins. Center, the cross-linking reaction with BS³ was performed as described above, but the reaction mixtures contained the following: left panel, 10 nM Kit-X and increasing concentrations of ¹²⁵I-SCF as indicated; middle panel, 4 nM ¹²⁵I-SCF and increasing concentrations of Kit-X as indicated; right panel, Kit-X, 4 nM ¹²⁵I-SCF, and increasing concentrations of unlabeled SCF as indicated. Lower, the cross-linking reaction with BS³ was performed as described for A, except that unlabeled SCF was used and Kit-X was detected by Western blotting. After termination of the cross-linking reaction, the samples were immunoprecipitated with Kit 94-IgG₁ monoclonal antibody, washed, separated on 7.5% SDS-polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with the rabbit antibody to Kit-X. Kit-X was detected by the Enhanced Chemiluminescence method (Amersham Corp.) using protein A coupled to horseradish peroxidase.

(Günther *et al.*, 1990). A secreted ectodomain of the PDGF receptor also undergoes ligand-induced dimerization (Duan *et al.*, 1991). However, the monomeric form predominated in the PDGF receptor case, unlike in the case of the Kit-X protein, where the dimeric form accounted for most of the radioactively labeled protein.

Inhibition of Autophosphorylation of the SCF Receptor by the Kit-X Protein—The ability of the recombinant Kit-X protein to compete with SCF for binding to the intact surface receptor (Fig. 5) suggested that Kit-X may antagonize the biochemical action of SCF. As receptor autophosphorylation on tyrosine residues is one of the earliest events in the signal transduction pathway of Kit, we examined the antagonism of this function. CHO cells that overexpress Kit (T18) were metabolically labeled with ortho[32 P]phosphate and then incubated for 15 min with 3.3 nM SCF in the presence of various concentrations of Kit-X. Subsequent immunoprecipitation with antibodies to phosphotyrosine, specific elution, and final precipitation with a rabbit antiserum to the Kit protein revealed the following (Fig. 7). No tyrosine phosphorylation of the receptor took place in the absence of SCF. Incubation with the ligand induced strong labeling of the receptor band, but this was gradually decreased when SCF was added in the presence of increasing concentrations of Kit-X. We therefore concluded that Kit-X is capable of inhibiting autophosphorylation of the Kit kinase following stimulation with SCF. This is very likely due to the inhibition of SCF binding to the cell-bound receptors.

Kit-X Inhibits Coupling of Kit/SCF Receptor to Signal Transduction Pathways—It has been previously shown that, upon ligand activation of the Kit tyrosine kinase, it couples to several signaling molecules including phosphatidylinositol 3'-kinase and the Raf1 protein kinase (Lev *et al.*, 1991; Rottapel *et al.*, 1991). We therefore asked whether the soluble Kit-X protein had the capacity to antagonize these functions. To measure coupling of the SCF receptor to phosphatidylinositol 3'-kinase we incubated T18 cells with 3 nM human recombinant SCF in the presence of increasing concentrations of Kit-X. Following 10 min of incubation at 22 °C, the Kit/SCF receptor was immunoprecipitated and assayed *in vitro* for associated phosphatidylinositol 3'-kinase activity. The results of this analysis (Fig. 8) are presented as an autoradiograph of the *in vitro* phosphorylated phosphatidylinositol (A panels) and by densitometric analysis of the autoradiograph (B panels). As is evident from the results, low (if any) association of phosphatidylinositol 3'-kinase with the SCF receptor could be seen in the absence of the ligand. Once incubated with SCF, the receptor coupled to phosphatidylinositol 3'-kinase activity, but this could be inhibited almost completely upon simultaneous incubation of the cells with the Kit-X protein. The extent of inhibition by Kit-X was found to be dependent on the concentration of the ligand used for cell

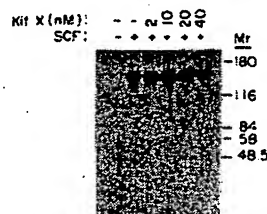


FIG. 7. Inhibition of tyrosine phosphorylation of SCF receptor by Kit-X. Quiescent T18 cells were metabolically labeled with ortho[32 P]phosphate for 4 h and then stimulated for 15 min at 22 °C with 3.3 nM recombinant SCF. Kit-X, at the indicated concentrations, was added together with SCF. The cells were solubilized, and tyrosine-containing proteins were immunoprecipitated with antibodies to phosphotyrosine. Specific elution was performed with phenyl phosphate, and the Kit/SCF receptor was reimmunoprecipitated with a rabbit antiserum to the carboxyl terminus of the protein (antibody 212). Washed immunocomplexes were separated on a polyacrylamide gel that was dried and exposed for 2 days to Kodak XAR film. The autoradiograph is shown, and the locations of molecular weight marker proteins are indicated.

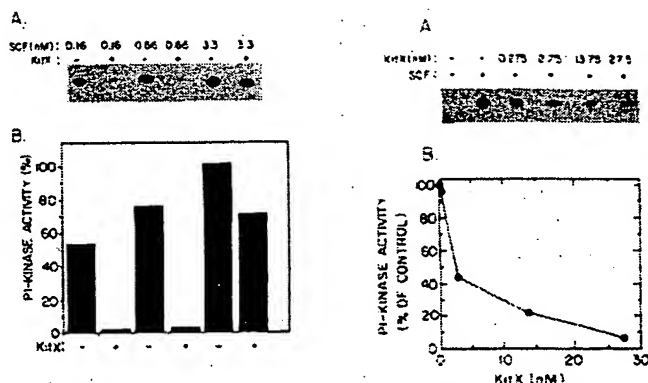


FIG. 8. Inhibition of SCF-induced phosphatidylinositol 3'-kinase activity by Kit-X. *Right*, quiescent T18 cells in 60-mm dishes were incubated with SCF (10 nM) in the presence of increasing concentrations of Kit-X, as indicated. After 10 min at 22 °C, the cells were solubilized, and the Kit/SCF receptor was immunoprecipitated with antibody 212 directed against the carboxyl terminus of the protein. Phosphatidylinositol (PI) 3'-kinase activity in the washed immunoprecipitates was assayed as described under "Materials and Methods," and the reaction products were separated on thin-layer chromatography plates. An autoradiograph of the phosphorylated phosphatidylinositol in each immunoprecipitate is given in A. Densitometric analysis of the autoradiograph is shown as the percentage of phosphatidylinositol 3'-kinase activity in the absence of Kit-X (B). *Left*, T18 cells were incubated with the indicated concentrations of SCF in the presence or absence of Kit-X (16 nM). The incubation with SCF, the subsequent determination of phosphatidylinositol 3'-kinase activity, and analysis of the results were done as described (right).

stimulation (Fig. 8, left). Thus, whereas 16 nM Kit-X inhibited (by >90%) the response to 0.16 nM SCF, the same concentration of Kit-X yielded only 30% reduction of the phosphatidylinositol 3'-kinase signal elicited by 3.3 nM SCF.

The Raf1 serine/threonine kinase undergoes phosphorylation in response to treatment of cells with a variety of mitogens (Rapp *et al.*, 1988). Both the receptors for PDGF (Morrison *et al.*, 1990) and colony-stimulating factor 1 (Baccarini *et al.*, 1990) induce phosphorylation and subsequent catalytic activation of the Raf1 kinase. The Kit/SCF receptor also couples to the Raf1 kinase upon ligand stimulation (Lev *et al.*, 1991). To determine if the Kit-X protein is capable of antagonizing the effect of SCF on the Raf1 kinase, we incubated T18 cells with SCF in the presence or absence of Kit-X and then immunoprecipitated the Raf1 protein with an antipeptide antiserum. As shown in Fig. 9, SCF induced a reduction in the electrophoretic mobility of the Raf1 protein, indicative of its hyperphosphorylation (Morrison *et al.*, 1989). Coincubation of the cells with SCF and Kit-X abolished the mobility shift of Raf1 (Fig. 9), thus revealing the capability of Kit-X to also antagonize this function of SCF.

DISCUSSION

The construction, expression, and initial biological characterization of a soluble form of the Kit/SCF receptor were described in this report. The recombinant protein, termed Kit-X, was properly expressed and heavily glycosylated by CHO cells. It was further secreted into the medium of these cells in a correctly folded form, as indicated by immunological cross-reactivity with the native surface receptor and by full retention of ligand recognition. The latter property apparently enabled the recombinant protein to antagonize three cellular responses to SCF, namely tyrosine autophosphorylation of the receptor, association with phosphatidylinositol 3'-kinase activity, and modification of the Raf1 protein kinase. Inter-

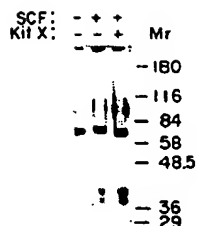


FIG. 9. Inhibition of SCF-stimulated modification of Raf1 by Kit-X. Growth-arrested T18 cells in 90-mm dishes were incubated with SCF (3.3 nM) for 10 min at 37 °C in the presence or absence of Kit-X (10 or 20 nM) as indicated. The cells were then solubilized, and the Raf1 protein was immunoprecipitated with a rabbit antiserum directed against a synthetic peptide. The resulting immunocomplexes were separated by gel electrophoresis, electrophoretically transferred onto nitrocellulose filters, and probed with the same antiserum to Raf1. Probing of the immunoblot was done by reaction with horseradish peroxidase conjugated to protein A and chemiluminescence-based detection followed by autoradiography. The Raf1 protein is the major 75-kDa band.

estingly, the truncated receptor underwent extensive dimerization upon binding to the ligand, thus raising the possibility that inhibition of SCF functions may be mediated by heterodimerization with surface receptors. The observed properties of Kit-X are analogous to the characteristics displayed by an extracellular domain of the PDGF receptor (Duan *et al.*, 1991). In addition, the previously reported ligand binding to an ectodomain of Kit fused to alkaline phosphatase (Flanagan and Leder, 1990) is also consistent with our results.

Kit-X displayed ligand affinity of ~1 nM, which therefore demonstrates that the transmembrane and cytoplasmic domains are not essential for the establishment of high affinity SCF binding. Other receptors, including the insulin receptor (Johnson *et al.*, 1989; Paul *et al.*, 1990; Schaefer *et al.*, 1990), the interleukin-1 receptor (Dower *et al.*, 1989), and the PDGF receptor (Duan *et al.*, 1991), also exhibit high affinity ligand binding when expressed as truncated proteins. In contrast, the ligand affinity of the EGF receptor is significantly reduced when the transmembrane and cytoplasmic domains are deleted (Weber *et al.*, 1984; Basu *et al.*, 1989; Günther *et al.*, 1990). Moreover, the isolated ectodomain of the EGF receptor, unlike the wild-type protein, displays a homogeneous population of relatively low affinity binding sites (Günther *et al.*, 1990), as do deletion mutants that lack only short portions of the carboxyl terminus (reviewed by Schlessinger (1988)). In contrast to Kit-X, the ectodomain of the EGF receptor does not undergo ligand-induced dimerization (Yarden and Schlessinger 1987b; Günther *et al.*, 1990). Since ligand affinity may be determined by the state of receptor oligomerization (Yarden and Schlessinger, 1987a; Boni-Schnetzler and Pilch, 1987), the possibility is raised that the ability of Kit-X to dimerize may be responsible for the full retention of ligand affinity.

The dimerization capability of Kit-X may, however, represent a passive function that is determined mostly by the dimeric nature of the ligand. Indeed, the recombinant SCF used in this study behaved, upon gel filtration, as a noncovalently held dimeric protein.³ Alternatively, the dimerization function is an intrinsic receptor function and, by inference, would be mediated by dimerization site(s) confined to the extracellular domain of Kit. These two mechanisms of receptor dimerization may be experimentally distinguishable. Ligand-mediated dimerization would result in a biphasic (bell-shaped) dimer formation as a function of ligand concentration. The data presented in Fig. 6 (*center and lower*) argue against this possibility as increasing ligand concentrations

did not favor the monomeric state. Nevertheless, more experiments will be needed for definite exclusion of the ligand-mediated mechanism of Kit dimerization.

The simplest interpretation of the antagonistic function of Kit-X is that the ligand was depleted by direct binding to the soluble receptor. Yet, an alternative mechanism would attribute the antagonism to nonfunctional heterodimers of a truncated Kit-X and a full-length receptor. This latter mechanism is apparently functional in the case of the EGF receptor on A431 cells (Basu *et al.*, 1989). Another example is the thyroid hormone receptor, where a nonhormone-binding protein was shown to inhibit thyroid hormone actions (Koenig *et al.*, 1989). Our attempts, however, to detect heterodimers of Kit-X and the SCF receptor by means of covalent cross-linking have so far been unsuccessful (data not shown).

Naturally occurring ectodomains of several receptors for hormones and lymphokines have been reported. For example, a developmentally regulated truncated form of the PDGF receptor was observed in embryonic stem cells (Vu *et al.*, 1989), and an alternative splicing product of the EGF receptor was identified in normal rat tissue (Petch *et al.*, 1990). The demonstration (in this study) that an ectodomain of the SCF receptor retains ligand binding and of the availability of antibodies to the extracellular domain (Fig. 3) may facilitate the survey of tissues and cell lines that potentially produce a soluble SCF receptor. The existence of such a protein is particularly intriguing in the case of SCF as a surface-bound form of this ligand is involved in cell migration and differentiation (Flanagan *et al.*, 1991).

The availability of a functional Kit-X protein may enable detailed analysis of ligand-receptor interactions, including the existence of a receptor dimerization site, the valency of ligand binding, and crystallization of the ligand-receptor complex. Besides the potential for research, the antagonistic function of Kit-X may be useful for interruption of normal or disease-associated biological actions of SCF. These may include hyperproliferative or neoplastic states of myeloid, neuronal, and germ cells in which SCF apparently plays a physiological role.

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